THE CHARACTERIZATION OF HUMIC SUBSTANCES IN SEAWATER

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ABSTRACT

Humic substances were isolated in gram quantities from seawater by a newly developed procedure of adsorption on a crosslinked polystyrene-divinylbenzene resin. The chemical and physical characteristics of both humic acid and fulvic acid fractions were studied.

The elemental composition, the acidimetric titration characteristics, the $^{13}C:^{12}C$ ratios, and the ultraviolet-visible, fluorescence, and infrared spectra were determined. Molecular weight distributions of coastal and Sargasso Sea fulvic acids were measured by gel permeation chromatography. Structural features were further investigated by both proton and carbon-13 nuclear magnetic resonance spectroscopy. In addition, the fulvic acids and their derivatives were analyzed by low and high resolution mass spectrometry and combined gas chromatography-mass spectrometry (GC-MS). Amino acids and organic solvent-soluble products in acid hydrolyzates were investigated. An array of biogenic hydrocarbons produced from fulvic acid by a new reduction scheme were characterized by GC-MS.

The structural features of seawater humic substances are complex. They are highly aliphatic, polyfunctional materials containing both polar and nonpolar moieties. Hydrolyzable amino acids constitute a low percentage of the nitrogen. Fatty acids and other lipids are important structural components. Seawater humic substances have significant structural differences from those of terrestrial origin; this seems to result mainly from the relatively low input of lignin to the marine environment and the differences between the physical environment of the soil and the sea.

A mechanism is proposed for the formation of seawater humic substances from amino acids, sugars and lipids. The effects and fate of humic substances in the sea are discussed.

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To Betty

and

My Parents

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TABLE OF CONTENTS

LIST OF FIGURES

List of Figures (Cont.)

LIST OF TABLES

INTRODUCTION

The sea is the second largest reservoir of organic carbon on Earth after the sediments. It contains approximately 800 billion tons; 2% of this is present as living organisms, 9% as particulate detritus and 89% as dissolved organic matter (Horne, 1969). The source of almost all the organic carbon in seawater is marine organisms with small inputs from terrestrial runoff and rain (Menzel, 1974) and possibly abiotic synthesis (Fripiat et al., 1972; Harvey et al., 1972). Simple organic compounds are transformed in seawater into complex materials referred to as aqueous humus (Skopintsev, 1972; Fotiyer, 1972) or Gelbstoff (Kalle, 1966). Organic materials which reach the sediments are subject to further chemical reactions. However, the reactions in seawater and surface sediments, the kinetics and the products of the transformations are only poorly understood.

The organic compounds in seawater have effects on biological, physical and geochemical processes in the sea. For example, primary productivity is influenced by the presence of dissolved organic carbon (Barber and Ryther, 1969; Sunda, 1975; Prakash and Rashid, 1968) and organic carbon may be an important source of energy for organisms living in deep waters (Craig, 1970 Wangersky, 1972). Organic matter in seawater modifies the air-sea interface. It is responsible for sea slicks, the foaming of seawater (Blanchard, 1964; Garrett, 1972), and the damping of capillary waves (Garrett, 1967 Barger et al., 1974). Organic compounds participate in processes at the sediment-water interface. Ion exchange (Rashid, 1969), calcite precipitation (Kitano and Hood, 1964; Chave and Suess, 1970) and the surface charge on particles

(Neihoff and Loeb, 1974) may be influenced by the organic matter in seawater.

The oxidation of organic matter affects the redox potential of seawater which, in turn, can have dramatic effects on biological and chemical processes (Breck, 1974). Under special conditions of limited circulation, complete utilization of oxygen can result in reducing environments such as the Cariaco Trench and Santa Barbara Basin.

In spite of the importance of organic carbon in seawater, little is known about how this material influences biological, physical and other chemical processes in the sea, what reactions occur during the initial stages of transformation and what products result from these transformations. About 10% by weight of the dissolved organic matter in seawater has been identified as common components of living organisms such as free and combined amino acids, carbohydrates, fatty acids, hydrocarbons, steroids, urea and glycolic acid (Wagner, 1969; Riley and Chester, 1971; Gagosian, 1975). Structural features and physical properties of the complex material referred to as aqueous humus or Gelbstoff are almost completely unknown.

Bulk Properties

Resistance

The resistance of seawater organic matter to bacterial degradation has been investigated by several workers (Kriss, 1963; Barber, 1968; Ogura, 1970; Ogura, 1972; Skopintsev, 1972). The results demonstrate that a fraction of the surface water organic matter and nearly all of the deep water organic matter is resistant to bacterial decomposition.

Isotope Ratios

Measurements of $^{13}C:^{12}C$ ratios of the dissolved and particulate organic matter in seawater (Williams, 1968; Williams and Gordon, 1970; Nissenbaum, 1974) demonstrate that ^{12}C is enriched during the formation of these materials from the organic matter of primary producers. The preservation of the lighter lipid fraction (Nissenbaum, 1974) relative to the more labile protein and carbohydrate fractions may be responsible.

Spectra

Investigations of the ultraviolet (uv) and visible (vis) absorption and fluorescence of surface seawater have been carried out by Kalle (1938, 1949, 1956, 1961, 1963, 1966), Jerlov (1955, 1968), and others (Armstrong and Boalch, 1961; Ogura and Hanya, 1952). These authors have shown that seawater displays a smoothly increasing absorption of visible and ultraviolet light with decreasing wavelength and displays a blue fluorescence upon irradiation with ultraviolet light. Both the absorption and the fluorescence decrease as the salinity of the seawater sample increases, but the fluorescence to absorption ratio increases. Without more detailed knowledge of the seawater organic matter composition, specific interpretation of these results is not feasible.

Kalle (1966) has observed that the ratio of absorbance at 420 nm to that at 665 nm increase as the salinity of the seawater increases. The value and increasing trend of this ratio with salinity has been interpreted by Kalle to indicate that the absorbing organic materials in seawater called

Gelbstoff are produced in seawater and have structural features similar to melanoidins formed as condensation products of amino acids and sugars. This interpretation is extremely speculative considering the lack of any supporting evidence.

Observations by Jerlov (1968) of high uv-vis absorbance in highly productive upwelling waters west of South America provide evidence that light absorbing substances are produced in seawater. In support of these findings are results of laboratory studies which demonstrate that light absorbing substances are produced in water of cultures of marine algae (Fogg and Boalch, 1958; Craigie and McLachlan, 1964; Yentsch and Reichert, 1961; Sieburth and Jensen, 1969; Rashid and Prakash, 1972).

Studies of Aqueous Humus

Several investigators have attempted to characterize fractions of the dissolved organic carbon isolated from seawater (Khaylov, 1968; Sieburth and Jensen, 1968; Kerr and Quinn, 1975). Khaylov (1968) isolated a fraction of coastal seawater organic matter by adsorption at the interface of seawater-chloroform emulsions. This material was base soluble and gel permeation chromatography demonstrated that its molecular weight extended to 200,000. From these results, Khaylov (1968) speculated that the material he isolated was proteinaceous in character.

Sieburth and Jensen (1968) isolated a fraction of the dissolved organic carbon from acidified coastal water collected 6 km west of Trondheim, Norway by absorption on nylon cloth. Analysis of basic eluents by two-dimensional paper chromatography using bis-diazotized benzidine spray reagents led these

investigators to assign a phenolic nature to the isolated organic matter. Materials isolated from river water by the same procedure showed different chromatographic patterns, suggesting that the source of the organic matter isolated from seawater was not river runoff.

Kerr and Quinn (1975) have isolated, by adsorption on charcoal, a fraction of the dissolved organic matter from coastal and Sargasso Sea surface waters. Ultraviolet and visible spectra of base eluents exhibited absorbance that increased smoothly towards shorter wavelength. The extinction of comparable base extracts of soil and bay sediments was higher than that of the Sargasso Sea sample. Therefore, the base soluble material isolated from seawater possesses fewer chromophores than comparable soil or sediment extracts. This indicates a less condensed character for the seawater base-soluble materials.

These investigations leave many gaps in our understanding of the origin, structure and properties of aqueous humus. For example, what fractions of the biologically produced organic matter are incorporated into the aqueous humus, how are these substances involved in biological, chemical and physical processes in the sea, and what fractions of the organic matter are ultimately incorporated into the sediments?

From the studies of Khaylov (1968), Sieburth and Jensen (1968), Jeffrey and Hood (1958) and Kerr and Quinn (1975) it is known that at least 30% of the uncharacterized organic matter in seawater is soluble in aqueous base. Base soluble organic materials are also important constituents of soils and sediments.

Soil and Sedimentary Humic Substances

Base soluble materials isolated from soils and sediments are defined as humic substances and have been studied extensively. (Soils: Stevenson and Butler, 1969; Kononova, 1966; Schnitzer and Khan, 1972; Sediments: Wakesman, 1933; Rashid and King, 1971, Ishiwatari, 1971; Nissenbaum and Kaplan, 1972; Bordovsky, 1965; Degens et al., 1964; Jackson, 1975.) Humic substances constitute the largest fraction of the organic matter in soils and modern sediments. They are thought to arise from plant and animal material by decomposition and resynthesis; both bacterial and non-biological processes may be involved. They are complex mixtures of variable molecular weight, oxygenated, polyfunctional molecules containing heteroatoms and metals, and phenolic, carboxylic, keto, amido, hydroxylic and quinoid functionality. Specific structural features are not well understood.

Soil and sedimentary humic substances show differences in elemental composition, specific density, functional groups, and products produced upon oxidative or reductive degradation which suggest differences in structure and a more aromatic character for the soil humic substances (Ishiwatari, 1971; Nissenbaum and Kaplan, 1972; Huc and Durand, 1974; Rashid and King, 1970; Ishiwatari, 1969). These investigations have demonstrated that humic substances are produced by decomposition and resynthesis in both soils and sediments, however, differences in the sources of organic matter and the properties of the environment influence the structural features of the final products.

Humic substances of soils and sediments are growth promoting agents for marine algae (Prakash and Rashid, 1968; Prakash et al., 1973) and terrestrial plants (Schnitzer and Poapst, 1967), chelators of metals (Koshy and Ganguly, 1969; Manning and Ramamoorthy, 1973; Martin et al., 1971; Rashid and Leonard, 1973), and solubilizing agents for hydrophobic substances in aqueous solution (Matsuda and Schnitzer, 1971; Boehm and Quinn, 1973). Humic substances may be precursors of kerogen in sediments (Degens et al., 1964; Brown et al., 1972; Huc and Durand, 1974).

This Investigation

The basic structural features of the large uncharacterized fraction of seawater dissolved organic matter must be elucidated if we are to understand its sources, mechanisms of formation and effects in the sea. This investigation concerns the characterization of a base soluble (humic substance) fraction of this material since it has been shown to constitute as much as 30% of the total.

The recovery of sufficient material to allow physical and chemical characterization studies was required. An isolation procedure was developed to obtain gram quantities of humic substances from seawater containing fractions of milligrams per liter in the presence of 35 grams per liter of salt. Humic substances were isolated from seawater well away from the influence of terrestrial runoff so that results of characterization represent humic substances of truly marine origin. Characterization included basic physical property analyses, as well as chemical analyses designed to provide

structural information. The results are compared to studies of humic substances from soils and sediments to aid in interpretation. The results are interpreted so as to provide information on structural features, possible precursors, and mechanisms of formation of the humic substances in seawater.

CHAPTER 1

Introduction

This chapter describes the procedure developed for the isolation of humic substances from seawater, the fractionation of the humic substances into humic acid (HA) and fulvic acid (FA) and analyses of bulk properties of these materials. Humic acids are operationally defined as organic materials which are soluble in aqueous base and insoluble in aqueous acid. Fulvic acids are soluble in both aqueous base and aqueous acid (Figure 1-1). Acid-base fractionation methods were developed by soil chemists to obtain more homogeneous subfractions of soil organic matter (Schnitzer and Khan, 1972); it is employed in this study for the same purpose.

The HA and FA were studied by ultraviolet (uv), visible (vis), fluorescence, and infrared (ir) spectroscopy, and their molecular weight distribution, elemental composition and $^{13}C/^{12}C$ ratios were determined. The equivalent weight of Sargasso Sea FA was determined by acidimetric titration. These data and comparison with data on soil and sedimentary humic substances, which have been more extensively studied, provided structural information on seawater humic substances.

The results presented in this chapter provide the background for the more extensive chemical investigations in Chapters 2 and 3.

Experimental

Northwestern Sargasso Sea surface and deep water samples were collected in February, 1973 (R/V CHAIN-111), and September, 1973 (R/V KNORR-33) (Figure 1-2,

Table 1-1). Coastal water samples were collected from a pier in three meters of water in Vineyard Sound, Massachusetts, U.S.A.

Surface seawater (6 m) was sampled by drawing water with vacuum through a 3/4 inch # 316 stainless steel pipe extended over the side of the ship and was collected in 55 gallon, # 316 stainless steel drums. Water samples contacted only stainless steel. The ship's sewer and garbage disposal were secured before arriving and while on station. The deep (1500 m) water sample (360 l) was collected by six successive lowerings of a 60 liter Bodman bottle (Bodman et al., 1961) and transferred to the 55 gallon drum.

All sampling equipment was cleaned prior to use with hot alkali, and successive washings of Micro detergent (International Products, Trenton, N. J.), water, 1 N HCl, 1 N NaOH, ethanol and distilled water. A final 1% NH_4OH rinse was checked by uv spectroscopy and showed no uv absorption higher than a reference solution. Prior to use, the sampling equipment was rinsed with seawater.

Isolation and Fractionation of Humic Substances

The seawater samples were acidified to pH 2 with 12 N HCl and passed through a 250 cc column (height:diameter = 10:1) of Amberlite XAD-2 resin (Rohm and Haas, Philadelphia, Pa.) (Rohm and Haas, 1972; Riley and Taylor, 1969) with glass wool plugs above and below to secure it and to prevent large particles from entering. Flow rates were less than two bed volumes per minute.

After use, the resin was frozen for storage and thawed before elution. The resin was then placed in a new column and salt was eluted with distilled

TABLE 1-1

Sargasso Sea Station Data

Station Location R/V CHAIN-111	Temperature (^{o}C)	Salinity (^{o}/oo)
39^{o}44.0'N, 64^{o}35.0'W	17.7	36.730
37^{o}32.8'N, 62^{o}52.1'W	22.1	36.618
35^{o}23.4'N, 60^{o}03.0'W	19.1	36.735
R/V KNORR-33 (Surface Water)		
35^{o}13.8'N, 63^{o}35.9'W	27.1	36.006
33^{o}39.0'N, 62^{o}20.4'W	27.1	36.330
32^{o}23.0'N; 59^{o}50.0'W	27.4	36.591
32^{o}18.5'N, 62^{o}59.4'W	27.8	36.258
(1500 m)		
32^{o}28.0'N, 59^{o}48.0'W	4.5	35.092

water to a negative silver nitrate test; no color was observed in this eluent. The humic substances were then eluted with 5 bed volumes of NH_4OH solution prepared fresh to pH 11.6 by dissolving reagent grade ammonia gas in $KMnO_4$ distilled water. The eluent was concentrated at 35°C to 25 ml on a rotary evaporator, acidified to pH 2, and extracted with methylene chloride to remove lipids. The humic substances were recovered from the aqueous solution by lyophilization. The fulvic acid fraction was obtained by dissolution in 0.01 N HCl; the insoluble humic acid was removed by centrifugation at 1800 g for 15 minutes. The humic acid was dissolved in NH_4OH, lyophilized to remove excess ammonia, and suspended in 0.01 N HCl. Lyophilization of the two fractions yielded the dry fulvic and humic acids in the protonated form.

The adsorption efficiency of the resin for fulvic acid was estimated by readsorption of the fulvic acid from 0.7 M NaCl (approximate seawater ionic strength) solution at pH 2 on an Amberlite XAD-2 column. Measurement of the uv absorbance of the solution entering and leaving the column at different flow rates was used to determine adsorption efficiencies. Efficiencies greater than 97% were observed at flow rates below 2 bed volumes per minute. Recovery from the column by base elution as described above was 73%.

An experiment was carried out to determine if nitrogen was incorporated into the fulvic acid by the NH_4OH elution procedure. Identical columns were used to isolate fulvic acid from identical coastal water samples. The first column was eluted with NH_4OH and the second column was eluted with NaOH of equal pH and ionic strength. Fulvic acid was recovered from the NH_4OH eluent as described above. NaOH was substituted in the isolation of fulvic

acid from the NaOH eluent. The C/N atomic ratios of the NaOH-eluted fulvic acid and NH_4OH-eluted fulvic acid were 15.1 and 13.4, respectively. The NH_4OH elution procedure was adopted in spite of this nitrogen increase to avoid contamination problems from desalting with ion exchange resins or losses of low molecular weight material while desalting by dialysis.

Preparation of the Resin

Raw Amberlite XAD-2 resin contains many impurities and requires extensive cleaning prior to use. Soxhlet extraction, first with acetonitrile then by several batches of benzene reduced blanks to nanogram levels within three weeks. The resin was checked for impurities by elution in a column with both 0.5 N NaOH and ethanol; the eluents were concentrated to 1/100 their volume and analyzed by uv. Also, the acidified base eluent and the water diluted ethanol eluent were extracted with hexane, the hexane was concentrated to 100 μl, and 5 μl was analyzed by gas chromatography on a 2% SE-30 column (Chromosorb W HP, 6 ft., temperature programmed, 75 to 300°C at 6°C/min). No peaks were observed with the clean resin eluent extract (detection limit 10 ng/component); the untreated resin showed a complex distribution of peaks.

During a two week exposure of Amberlite XAD-2 resin to 0.01 N HCl at room temperature in the light less than 5 µg of two compounds were formed. These compounds were identified (by methylation and GC-MS) as benzoic acid and phthalic acid. It is likely that these are products of oxidation of the Amberlite XAD-2 resin, a styrene-divinylbenzene polymer.

Spectroscopy: UV-VIS absorption spectra were measured in distilled water with a Cary model 14 spectrophotometer.

Ir spectra were obtained on a Perkin-Elmer Model 337 grating infrared spectrophotometer.

Fluorescence analyses were carried out on a Perkin Elmer model MPF-3L fluorescence spectrophotometer. Sample concentrations in distilled water were used such that absorbance was less than 0.05 at the excitation and fluorescence emission wavelengths to avoid non-linearity, quenching and self absorption. Fluorescence maxima as a function of excitation wavelength were determined by holding the emission monochromator at the fluorescence maximum while scanning the excitation monochromator from 200 to 400 nm. The following standard conditions were used to make relative measurements: an excitation wavelength of 313 nm was chosen and fluorescence intensity of 1.0 ppm (μg/ml) sample solutions was compared to that of a 1.0 ppm quinine sulfate (Matheson, Coleman and Bell Chemicals) solution at their fluorescence maxima.

$^{13}C/^{12}C$ Ratios: The $\delta^{13}C$ values of the CO_2 gas evolved in burning 1 mg of solid sample at 800°C over CuO was determined with a Nuclide mass spectrometer (State College, Pa.). $\delta^{13}C$ is defined as:

$$\left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{standard}} - 1 \right] \times 1000.$$

The National Bureau of Standards Norite sample 20 served as the standard but values are reported relative to Chicago Pee Dee Belemnite.

Elemental Analyses: Duplicate analyses for C, H, N, S, and ash were carried out by Galbraith Laboratories, Inc., (Knoxville, Tenn.) on the fulvic acid sample from the Sargasso Sea surface water.

Acid Titration: A titration of fulvic acid isolated from the Sargasso Sea was carried out in carbonate-free NaOH solution with standardized HCl (0.0975 N). The titration curve of the sample solution is compared to that of a blank NaOH solution.

Molecular Weight Distributions: The molecular weight distributions were determined by gel permeation chromatography (GPC) on Sephadex G-10, G-15 and G-25 gels (Pharmacia Fine Chemicals, Inc.). The eluent was 0.05 M NH_4HCO_3 (pH 7.1). Samples (10 mg) were dissolved in 5 ml of buffer and each chromatogram was run on a new sample. Columns were 2.5 cm in diameter and 80 to 100 cm long. The exclusion volume of each column was determined by chromatographing a 5 ml sample of Blue Dextran 2000 (molecular weight 2,000,000). Sample elution was monitored with a Gilford in-line absorptiometer (Oberlin, Ohio) at 280 nm. Five milliliter fractions were collected for measurements of absorption spectra.

Total Organic Carbon Analysis: The organic carbon content of unfiltered acidified seawater samples was determined by the method of Menzel and Vaccaro (1964).

Results

Recoveries of humic acid and fulvic acid from the seawater samples, the HA:FA ratios, the percent of the total organic matter (TOM) represented by these fractions, and the absolute amounts isolated in µg/l are presented in Table 1-2.

The *uv-vis absorption* of both the coastal and Sargasso Sea fulvic acid increases smoothly with decreasing wavelength except for a slight shoulder

TABLE 1-2

Recovery of Humic Substances from Seawater

Sample	Water Volume (l)	Total (HA + FA) (mg)	HA:FA Ratio	% of DOC	Absolute Recovery (µg/l)
R/V CHAIN-111					
Surface	1150	153.6	0:100	5.1	134
R/V KNORR-33					
Surface	2000	287.2	3:97	4.9	144
Deep	350	66.2	22:78	22.5	189
Pier - Vineyard Sound					
April, 1973	200	35.7	15:85	4.5	179
June, 1973	600	122.7	16:84	3.7	205

at 280 nm in the Sargasso sample (Figure 1-3). The ratio of absorbance at 420 nm to that at 665 nm (E 420/665) is lower for the Sargasso Sea fulvic acid than for the coastal fulvic acid. Light extinction on a unit weight basis is dramatically lower at all wavelengths for the sample from the Sargasso Sea (Table 1-3).

Fluorescence spectra of Sargasso Sea and coastal surface water fulvic acids are presented in Figure 1-4. A single fluorescence maximum at 405 nm is observed for the Sargasso sample while a primary fluorescence maximum at 415 nm and a secondary maximum at 437 nm is observed for the coastal sample (Figure 1-4). Single excitation maxima at 332 nm and 325 nm were observed for the Sargasso and coastal samples, respectively. Higher concentrations of 250 µg/ml were used to eliminate interference of the water Raman band for qualitative display in Figure 1-4. Sample fluorescence intensity compared to the quinine standard with dilute sample solutions (10 µg/ml) are presented in Table 1-4, together with fluorescence intensity relative to absorbance of the sample at 313 nm and 420 nm.

The *ir spectra* of Sargasso Sea surface water fulvic acid and coastal water fulvic and humic acid (Figure 1-5) all display broad absorption in the O-H and N-H stretching region characteristic of acids, alcohols, amines and amides (2900 to 3600 cm^{-1}). The absorption in the C-H stretching region (2800 to 3100 cm^{-1}) is partially masked by the acid O-H stretching but still evident in all spectra. The broad absorption at 1700 cm^{-1} is due to C=O stretching of carboxylic acids and other carbonyl groups. Strong absorption is also observed in the C-H bending region (1350 to 1480 cm^{-1}) and C-O, C-N, and C-C stretching region (900 to 1300 cm^{-1}).

TABLE 1-3

UV-VIS Extinction Coefficients ($l\ g^{-1}\ cm^{-1}$)

	Fulvic Acid Sample	
Wave Length (nm)	Sargasso Sea	Coastal Water
240	4.38	10.75
250	2.30	8.89
260	1.59	7.89
270	1.32	7.29
280	1.04	6.21
290	0.64	5.08
300	0.45	4.29
350	0.40	1.99
400	0.07	0.79
420	0.05	0.50
500	0.03	0.16
665	0.01	0.06
E 420/665:	4.8	8.2

TABLE 1-4

Fluorescence of Seawater Fulvic Acid

Fulvic Acid Sample	*Fluorescence Intensity	**Fluorescence:Absorbance Ratio 313 nm	420 nm
Sargasso Sea	1.31×10^{-3}	3.0	26.0
Coastal Water	4.82×10^{-3}	1.3	9.6

*Fluorescence intensity of 1 ppm sample solution relative to 1 ppm quinine sulfate solution - excitation wavelength 313 nm.

**Fluorescence intensity divided by absorbance (1 mg^{-1} cm^{-1}) at wavelength indicated.

The two fulvic acid ir spectra have absorptions at 1560 cm^{-1} which is absent in the humic acid spectrum (Figure 1-5). This band is characteristic of carboxylic acid anions, monosubstituted amides or primary amines.

The $\delta^{13}C$ values of Sargasso Sea surface water fulvic acid, coastal water fulvic acid and coastal water humic acid are -22.79 °/oo, -23.72 °/oo and -22.78 °/oo, respectively.

Table 1-5 presents the *elemental composition* and atomic ratios of Sargasso Sea surface water fulvic acid along with typical elemental compositional ranges of soil and marine sedimentary fulvic acids.

No inflections in the *titration* curve (Figure 1-6) nor secondary maxima in the derivative plot (Figure 1-6) are observed. An equivalent weight of 473 g/eq is calculated from the titration data.

The *gel permeation chromatograms* of Sargasso Sea and coastal water fulvic acid are presented in Figure 1-7 and 1-8. Also shown are the Blue Dextran 2000 chromatograms (cross-hatched areas) which have been normalized to the sample curves. The molecular weight distributions calculated from the GPC data for the two samples are presented in Figure 1-9. These distributions are calculated from the fraction of the sample excluded from each gel (equivalent to the cross-hatched area) and the fraction of the sample retained by that gel, (equivalent to the remaining area under the curve). The calculation requires the assumption of a constant extinction coefficient over the entire GPC run, though elution curves at 280 nm and 420 nm give evidence of variations in the relative extinctions during the GPC run. However, molecular weight distribution calculations using either wavelength agree within 4%. The assumption is expected to bias the results slightly towards higher molecular weight.

TABLE 1-5

Elemental Composition of Sargasso Sea Fulvic Acid
(Ash: 3.37%)

%C	%H	%N	%O	%S	H/C
49.98	6.76	6.40	36.40	0.46	1.61
Marine Sedimentary Fulvic Acid (Rashid and King, 1970)					
46.2-48.7	6.0-6.6	3.4-5.2	41.8-44.3	--	1.49-1.70
Soil Fulvic Acid (Schnitzer and Khan, 1972)					
42.5-50.9	3.3-5.9	0.7-2.8	44.8-47.3	0.3-1.7	0.77-1.64

Weighing of the small GPC fractions in the presence of large amounts of buffer salt was not feasible.

An uncertainty exists concerning the exclusion limits of the gels; therefore, molecular weight ranges are given as Sephadex gel grades. Calibration of the Sephadex G-10, G-15 and G-25 gels with proteins and polysaccharides yields exclusion limits of 700, 1500 and 5000, respectively. However, according to Schnitzer and Skinner (1968) these limits are too high when applied to soil fulvic acid and, therefore, may be high when applied to seawater humic substances.

All samples are eluted within one column volume and upon rechromatography of narrow elution fractions, elution again takes place within the same narrow band; this demonstrates that gel-solute interactions are not interfering with molecular size separation.

Discussion

The adsorption procedure developed in this study allows the convenient isolation of gram quantities of dissolved organic matter (DOM) from tons of seawater containing kilograms of salt. Between 4 and 23% of the total organic carbon in seawater is recovered as humic substances depending on the sample origin (Table 1-2). The stability of the divinylbenzene-styrene polymer resin (Amberlite XAD-2) allows organic blanks to be reduced to levels not attainable with charcoal or polyamide resins previously used (Sieburth and Jensen, 1968; Kerr and Quinn, 1975). The elution procedure avoids desalting of the final isolates.

Adsorption of humic substances on Amberlite XAD-2 resin is by simple hydrophobic bonding to the polyaromatic hydrocarbon resin surface. Only hydrophobic compounds or compounds containing hydrophobic moieties are adsorbed.

Hydrophilic compounds such as amino acids, sugars, etc. are not adsorbed by the resin (Rohm and Haas, 1972). Humic acids, because of their higher molecular weight and lower oxygen content, are much less water-soluble and more hydrophobic under acidic conditions than fulvic acids and would be expected to adsorb to XAD-2 more efficiently. Fulvic acids containing more hydrophobic moieties would be adsorbed on XAD-2 in preference to more hydrophilic fulvic acids. Therefore, a low recovery of humic acid (high FA:HA ratio) is evidence that low levels of humic acids are present in the seawater sampled. On the other hand, low recoveries of fulvic acids may result from either low levels in the seawater or a smaller fraction of fulvic acids containing sufficient hydrophobic character for adsorption.

In spite of the limited number of samples, it is interesting to speculate on variations in recoveries of humic substances and on the FA:HA ratios observed in different samples.

The highest (97:3) and lowest (78:22) FA:HA ratios are observed in the Sargasso Sea surface and deep water respectively, although the absolute recoveries in µg/l are similar (Table 1-2). These observations suggest that the transformation of fulvic acid to humic acid is occurring over time since the deep water contains organic matter which is older than surface water organic matter (Williams et al., 1969). The transformation of fulvic acid to humic acid in soils and sediments is suggested by several studies (Kobo and Tatsukawa, 1961; Nissenbaum and Schallinger, 1974; Nissenbaum, 1974).

The small humic substance fraction of the TOC (Table 1-2) in the Sargasso Sea surface water sample (5%) compared to that of deep water (23%) may be

the result of a large fraction of hydrophilic, non-humic substances freshly produced by biological productivity in the surface waters.

In coastal water samples, the low humic substance fraction of the TOC may also indicate that a large fraction of the TOC is present as hydrophilic and non-humic organic substances produced by biological productivity. In addition, adsorption and subsequent deposition of the more hydrophobic dissolved materials with the high particulate load in coastal waters will leave less dissolved material in solution which can be adsorbed by the Amberlite XAD-2 resin. The relatively low FA:HA ratio (85:15) in coastal water compared to the Sargasso Sea surface water sample, may result from the short water column in which humic acid fluxes from bottom sediments or bacterial or surface catalyzed conversion of FA to HA on the more abundant particulate matter can affect the dissolved humic substance composition. Terrestrial runoff does not seem to contribute since the $\delta^{13}C$ values of the coastal water humic substances (-22.7 to -23.8 $^{o}/oo$) are within the range of $\delta^{13}C$ values of marine sedimentary humic substances (Nissenbaum and Kaplan, 1972), dissolved organic matter in open ocean seawater (Williams, 1968), and very close to the value obtained for the Sargasso sea surface water fulvic acid (-22.8 $^{o}/oo$). Terrestrial humic substances typically have $\delta^{13}C$ values between -25 and -29 $^{o}/oo$ (Nissenbaum and Kaplan, 1972).

The elemental composition of the Sargasso Sea surface water fulvic acid is compared to that of fulvic acids isolated from soil and marine sediments in Table 1-3. The oxygen content is low and the H:C atomic ratio is high. The sulfur content is typical of soil fulvic acid values but low when compared to

fulvic acids from marine sediments. The nitrogen content is within the range of marine sedimentary fulvic acids but much higher than that reported for soil fulvic acids. The small ammonia uptake during isolation of the fulvic acid does not affect this interpretation.

The low oxygen content and high H:C ratio suggests fewer functional groups and less unsaturation in the seawater fulvic acid. This is supported by the lower light absorbance, especially in the visible range (Table 1-3; Figure 1-3), when compared to that of soil and sedimentary fulvic acid.

The low sulfur content observed for the fulvic acids from seawater and soil may reflect the low abundance of organic sulfur in plant and animal material, while the high sulfur values observed in marine sedimentary fulvic acid may result from diagenetic processes in reducing sediments (Nissenbaum and Kaplan, 1972).

High nitrogen functionality indicated by the abundant nitrogen in the seawater fulvic acid is supported by the ir absorption at 1560 cm^{-1} characteristic of secondary amides, amines or carboxylate anions (Figure 1-5). Absorption in this region is not observed in soil fulvic acid ir spectra but is prominent in ir spectra of lacustrine sedimentary humic substances (Ishiwatari, 1967). Ishiwatari observed a correlation between the absorbance at 1540 cm^{-1} and the nitrogen content presumably in amide functions of humic substances. However, carboxylate anions in amphoteric species also absorb in this region; their abundance would depend on the presence of amino cations and, therefore, nitrogen content. The 1560 cm^{-1} absorption in the fulvic acid spectra and its absence in the humic acid spectrum (Figure 1-5) supports that explanation since solubility in acid would be enhanced by the amphoteric property.

Except for the 1560 cm^{-1} absorption discussed above, the ir spectra of seawater humic substances show characteristics similar to those of soil and sedimentary humic substances. Complex and broad absorption bands suggest complex mixtures of polyfunctional organic molecules.

Complex mixtures of functional groups is also indicated by the lack of inflections on the main titration curve and lack of secondary maxima on the derivative plot (Figure 1-6) of seawater fulvic acid. The wide range of pK_a values may indicate polyacidic (Stumm and Morgan, 1970, p. 482) or mixtures of amphoteric structures. In contrast, soil humic substances display inflections on the titration curve which become more prominent in derivative plots (Borggaard, 1974); discrete groupings of pK_a's characteristic of carboxylic acid and phenolic functionality are indicated.

The equivalent weight of 473 g/eq determined for the Sargasso Sea surface water fulvic acid and high oxygen content (approximately 10 oxygens/eq; Table 1-5) indicate that most of the oxygen in the fulvic acid is present as non-acidic functional groups such as alcohols, ethers, amides and esters.

The $\delta^{13}C$ values of the seawater humic substances (-22.7 to -23.8 $^{o}/oo$) fall within those of humic substances isolated from marine sediments (Nissenbaum and Kaplan, 1972) and those of seawater dissolved and particulate organic carbon (Williams, 1968; Williams and Gordon, 1970). Continental humic substances have $\delta^{13}C$ values between -25 and -29 $^{o}/oo$ (Nissenbaum and Kaplan, 1972). This supports the assumption of marine sources for the seawater humic substances.

Most of the fulvic acid isolated from the Sargasso Sea has a molecular weight less than 700, with none extending above 5,000 (Figure 1-8). The coastal water fulvic acid molecular weight distribution is only slightly higher. In contrast, soil fulvic acid has molecular weights extending to 10,000 (using the same criteria) (Schnitzer and Skinner, 1968) or higher (Kononova, 1966). Sedimentary humic substances have molecular weights extending to 2,000,000 (Rashid and King, 1969).

Condensation reactions are less likely to occur in the dilute solution of seawater than in sediments or soils where organic matter is concentrated on particle surfaces. The lower molecular weight range observed for seawater fulvic acid may result from fewer condensation reactions in the extremely dilute solution existing in the sea.

From the uv-vis and fluorescence measurements, it is apparent that the uv-vis absorption and fluorescence intensity of the Sargasso Sea fulvic acid is less than that of the coastal fulvic acid. However, the fluorescence intensity: uv-vis absorbance ratio is higher for the Sargasso fulvic acid. These same general features have been observed for absorbance and fluorescence of total seawater by Kalle (1966) and, although quantitative comparison is not possible, the data suggests that the fulvic acid fraction of the TOC is partially responsible for Kalle's observations.

Low E 420/665 values and high light absorption have been used to indicate a high degree of condensation of humic substances (Nissenbaum and Kaplan, 1972; Kalle, 1966). However, for seawater humic substances both low light absorbance and low E 420/665 ratios are observed (this work; Kerr and Quinn, 1975) suggesting a low degree of condensation in spite of low E 420/665 ratios.

Kalle (1966) has observed that open ocean seawater has a higher E 420/665 value than coastal seawater. However, humic substances from the Sargasso Sea have a lower E 420/665 than humic substances of coastal water (this work; Kerr and Quinn, 1975). This indicates that other fractions of the TOC are responsible for this observation of Kalle's.

Summary

(1) A method was developed for isolating gram quantities of dissolved organic matter from seawater for chemical and physical analyses.

(2) The bulk properties of seawater fulvic and humic acid are summarized in Table 1-6.

(3) The marine origin of seawater humic substances is indicated by significant differences in compositional and spectroscopic characteristics from those of terrestrial humic substances.

(4) The low molecular weight range of seawater fulvic acid seems to result from the dilute solution in seawater which decreases the rate of intermolecular reactions.

(5) Differences between seawater and terrestrial humic substances are most likely the result of differences in sources of organic matter from which the humic substances are formed.

TABLE 1-6

Properties of Seawater Humic Substances

Elemental Composition: Sargasso Sea fulvic acid	a) C: 49.98; H: 6.40; O: 36.40; S: 0.46 b) H/C = 1.61 c) Stoichiometric formula: $C_9H_{15}O_5N$
UV-VIS Spectrum:	a) Smoothly increasing absorption with decreasing wavelength b) E420/665: 8.2 coastal water fulvic acid 4.8 Sargasso Sea fulvic acid c) Extinction of coastal greater than Sargasso Sea fulvics
Fluorescence Spectra:	a) Excitation maxima: Sargasso Sea fulvic acid: 332 nm Coastal water fulvic acid: 325 nm b) Fluorescence maxima: Sargasso Sea fulvic acid: 405 nm Coastal water fulvic acid: primary 415 nm secondary 437 nm c) Fluorescence intensity per unit weight: Sargasso Sea fulvic acid less than coastal water fulvic acid
IR Spectrum:	a) Broad absorptions characteristic of complex mixtures b) Similar to fulvic acids of other environments c) Absorption at 1560 cm^{-1} in fulvic acids - not in humic acid
$\delta^{13}C$ (PDB)	a) Sargasso Sea fulvic acid: -22.79°/oo b) Coastal water fulvic acid: -23.72°/oo c) Coastal water humic acid: -22.78°/oo
Acid Titration: Sargasso Sea fulvic acid	a) Smooth curve indicates wide range of pK_a's b) Equivalent weight = 473 g/equivalent
M. W. Distribution:	a) Coastal water fulvic acid: 55% less than 700 81% less than 1500 99% less than 5000 1% greater than 5000 b) Sargasso Sea fulvic acid: 73% less than 700 84% less than 1500 100% less than 5000

Figure 1-1. Fractionation of humic substances.

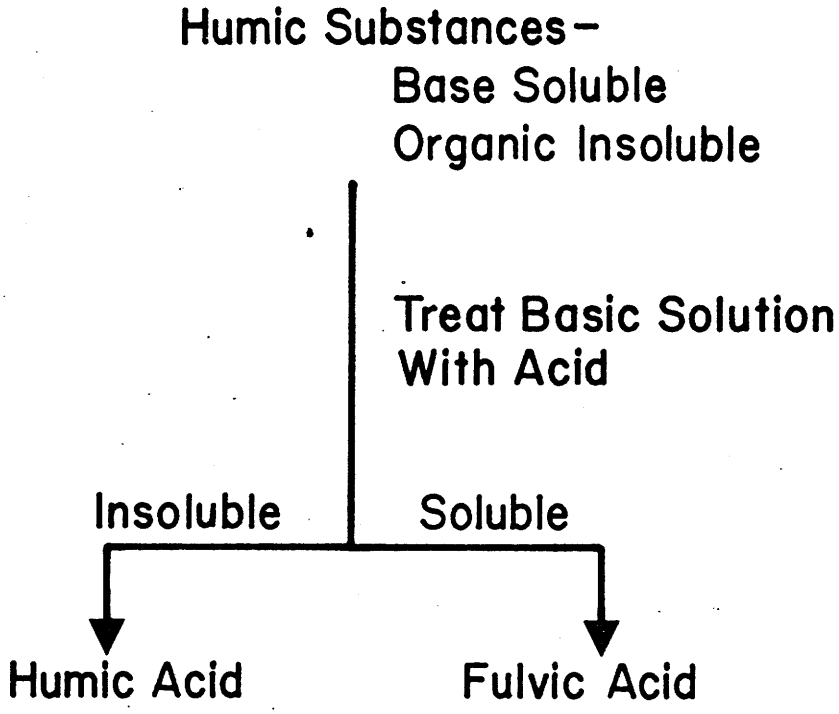

Humic Substances–
Base Soluble
Organic Insoluble
Treat Basic Solution
With Acid
Insoluble
Soluble
Humic Acid
Fulvic Acid

Figure 1-2. Sargasso Sea sampling stations on R/V CHAIN cruise 111 (O), and R/V KNORR cruise 33 (surface sample ■; surface and deep sample ▲).

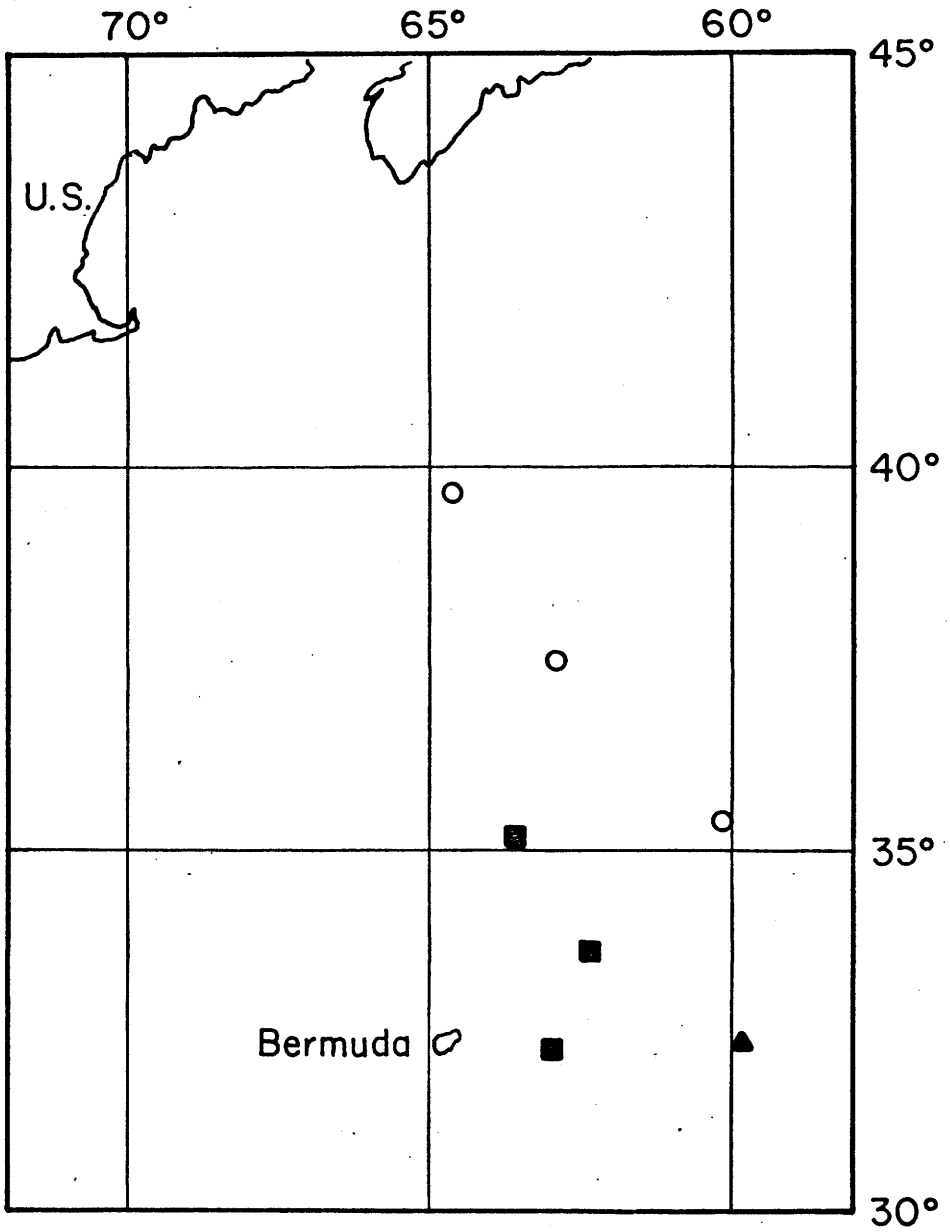

70°
65°
60°
45°
U.S.
40°
35°
Bermuda
30°

Figure 1-3. UV-VIS absorbance spectra of Sargasso Sea surface water fulvic acid (FASS-1) and coastal water fulvic acid (FACP-4).

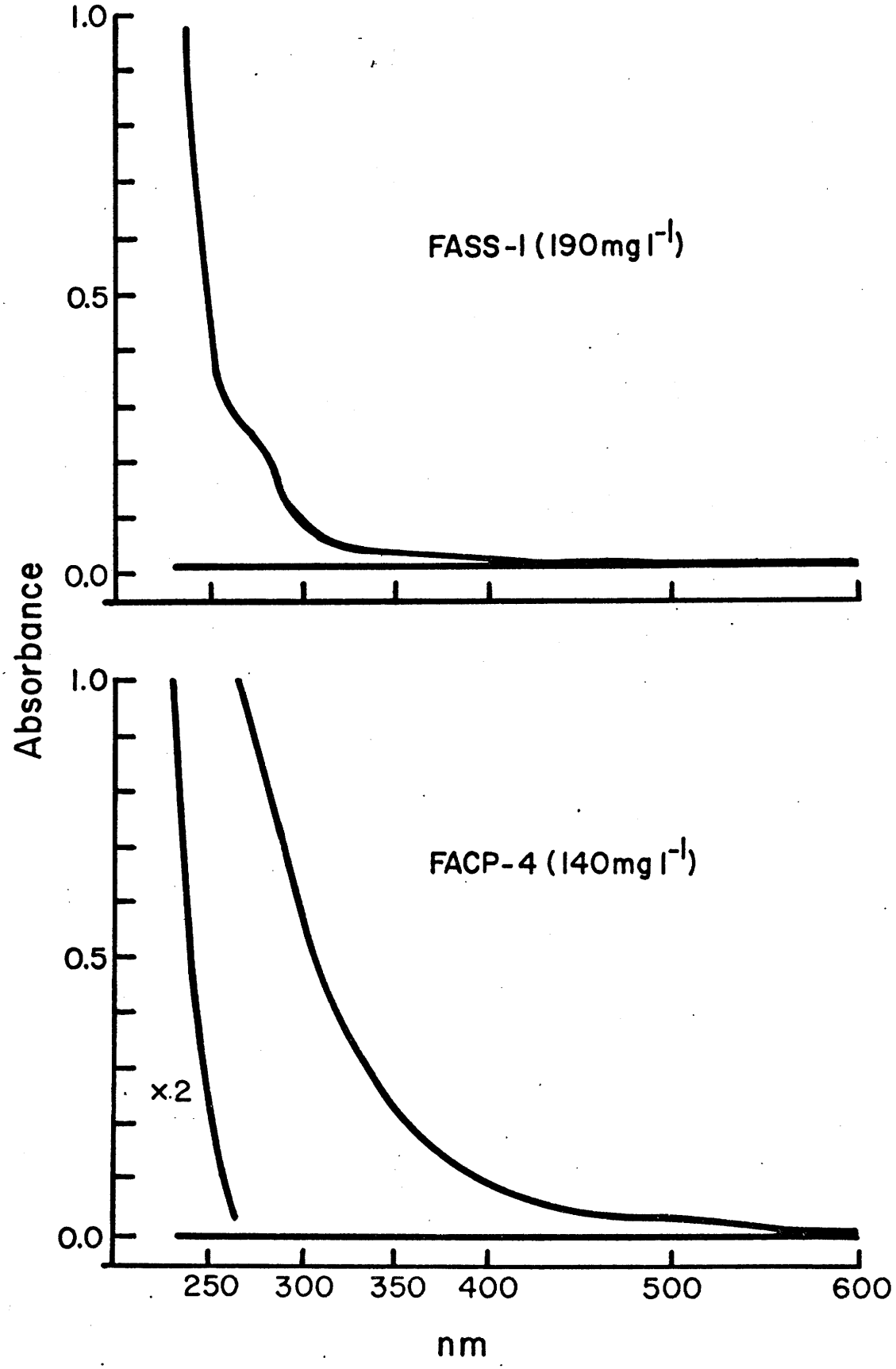

1.0
0.5
0.0
FASS-1 (190mg l⁻¹)
Absorbance
1.0
0.5
0.0
FACP-4 (140mg l⁻¹)
x.2
250
300
350
400
500
600
nm

Figure 1-4. Fluorescence spectra of Sargasso Sea surface water fulvic acid (A) and coastal water fulvic acid (B).

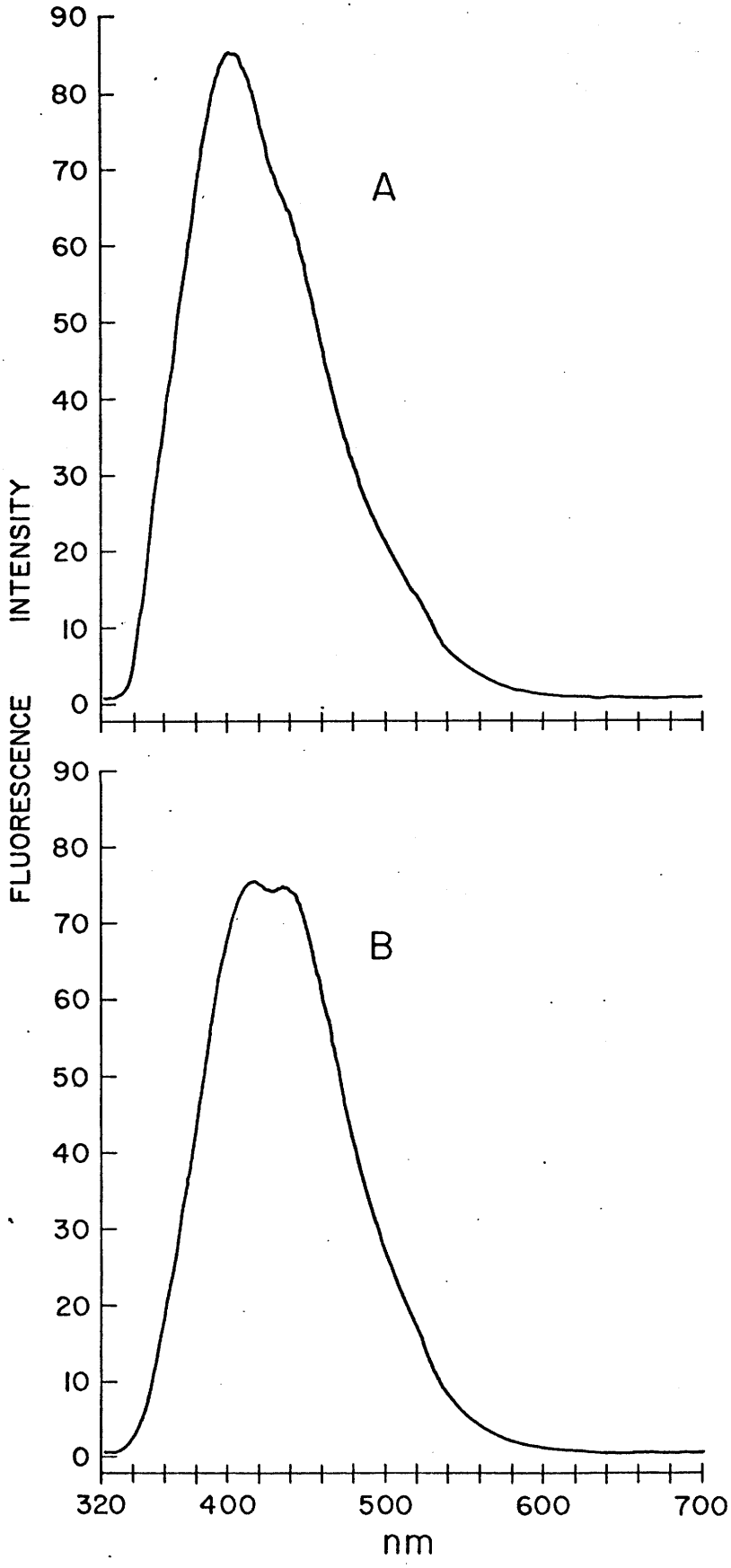

FLUORESCENCE INTENSITY
90
80
70
60
50
40
30
20
10
0
A
B
320
400
500
600
700
nm

Figure 1-5. Infrared spectra of Sargasso Sea surface water fulvic acid (A), coastal water fulvic acid (B), and coastal water humic acid (C).

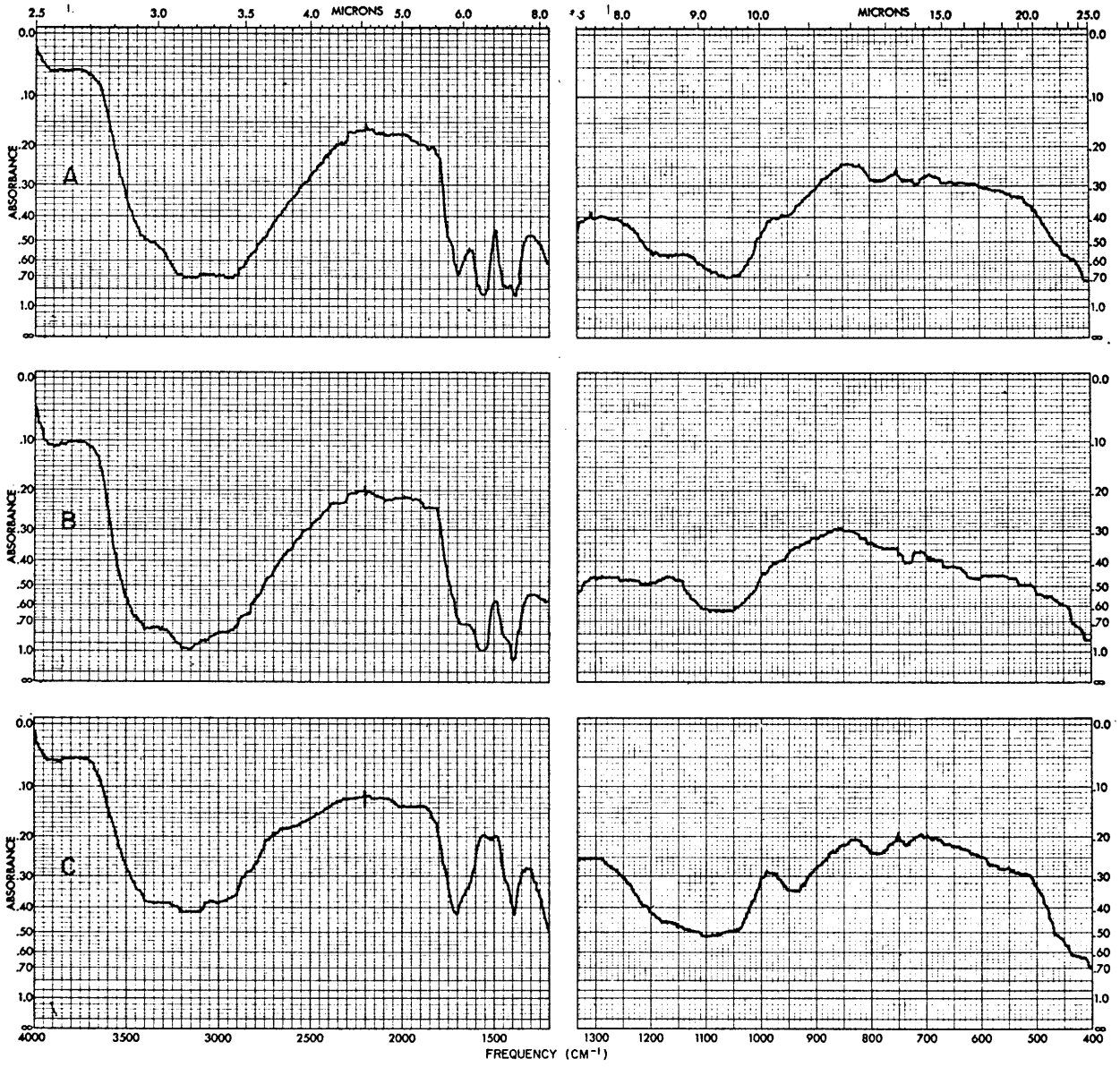

2.5
3.0
3.5
4.0
MICRONS
5.0
6.0
8.0
7.5
8.0
9.0
10.0
MICRONS
15.0
20.0
25.0
ABSORBANCE
A
B
C
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
4000
3500
3000
2500
2000
1500
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM⁻¹)

Figure 1-6. Acidimetric titration of Sargasso Sea surface water fulvic acid (upper plot, B) compared to background curve (upper plot, A), and derivative plot of acidimetric titration curve (lower plot).

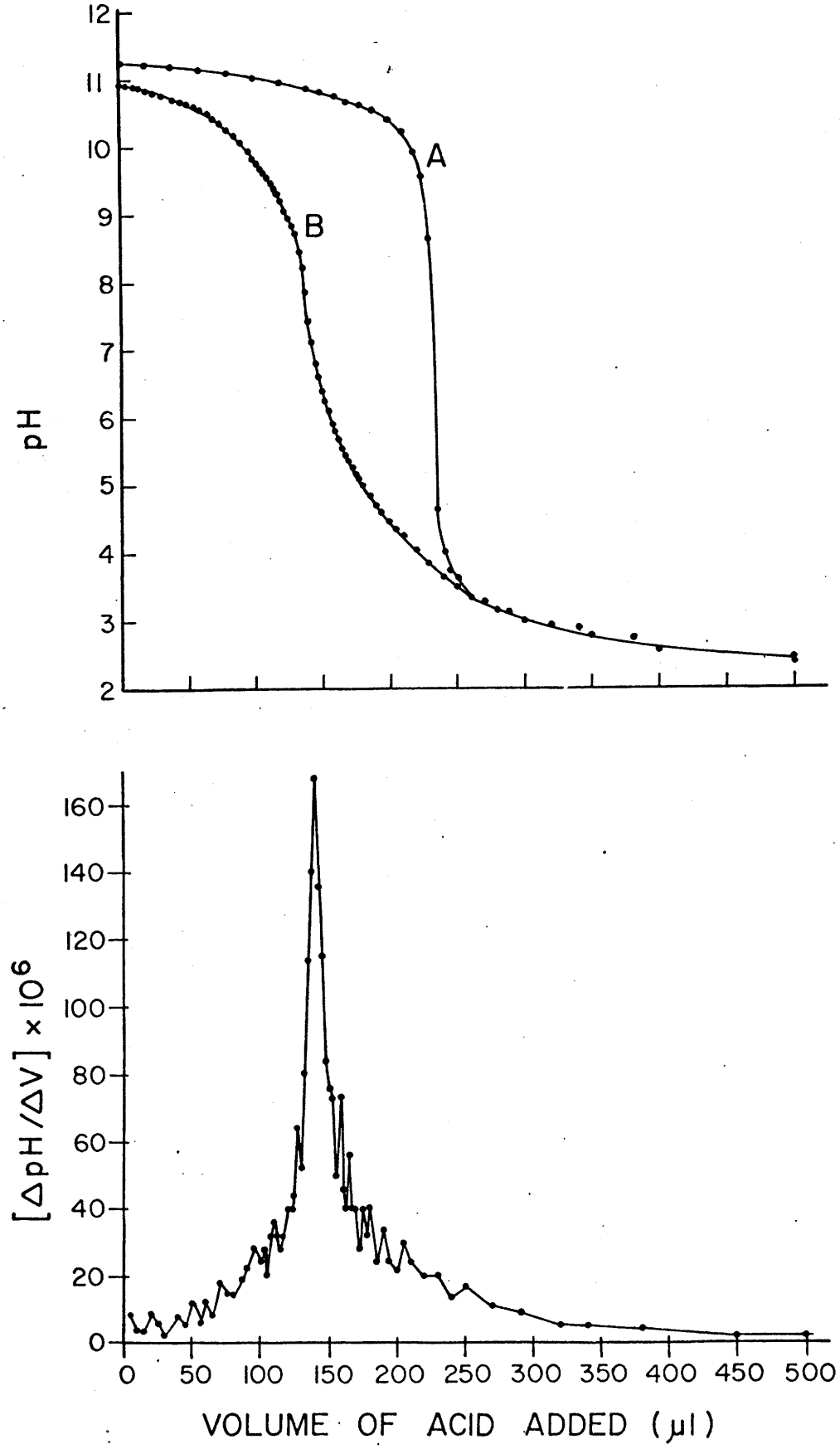

12
11
10
9
8
7
6
5
4
3
2
pH
A
B
160
140
120
100
80
60
40
20
0
[ΔpH/ΔV] x 10⁶
0
50
100
150
200
250
300
350
400
450
500
VOLUME OF ACID ADDED (μl)

Figure 1-7. Gel permeation chromatograms for Sargasso Sea surface water fulvic acid. The Sephadex gel grades are indicated (G-number) and areas under Blue Dextran 2000 chromatograms (exclusion volume) are cross-hatched.

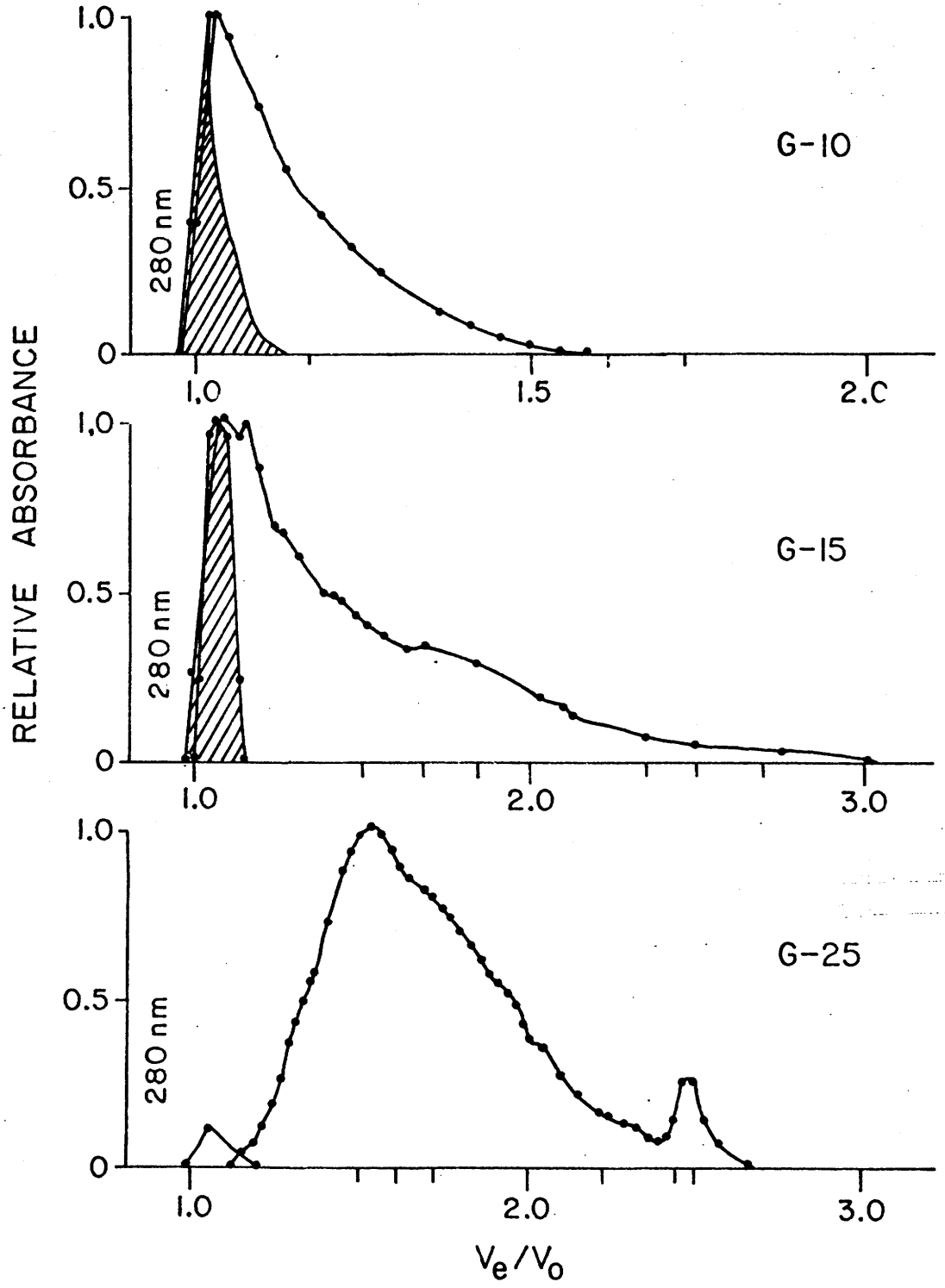

RELATIVE ABSORBANCE
1.0
0.5
0
280 nm
G-10
1.0
1.5
2.0
G-15
2.0
3.0
G-25
V_e / V_o

Figure 1-8. Gel permeation chromatograms for coastal water fulvic acid. The Sepadex gel grades are indicated (G-numbers) and areas under Blue Dextran 2000 chromatograms (exclusion volume) are cross-hatched.

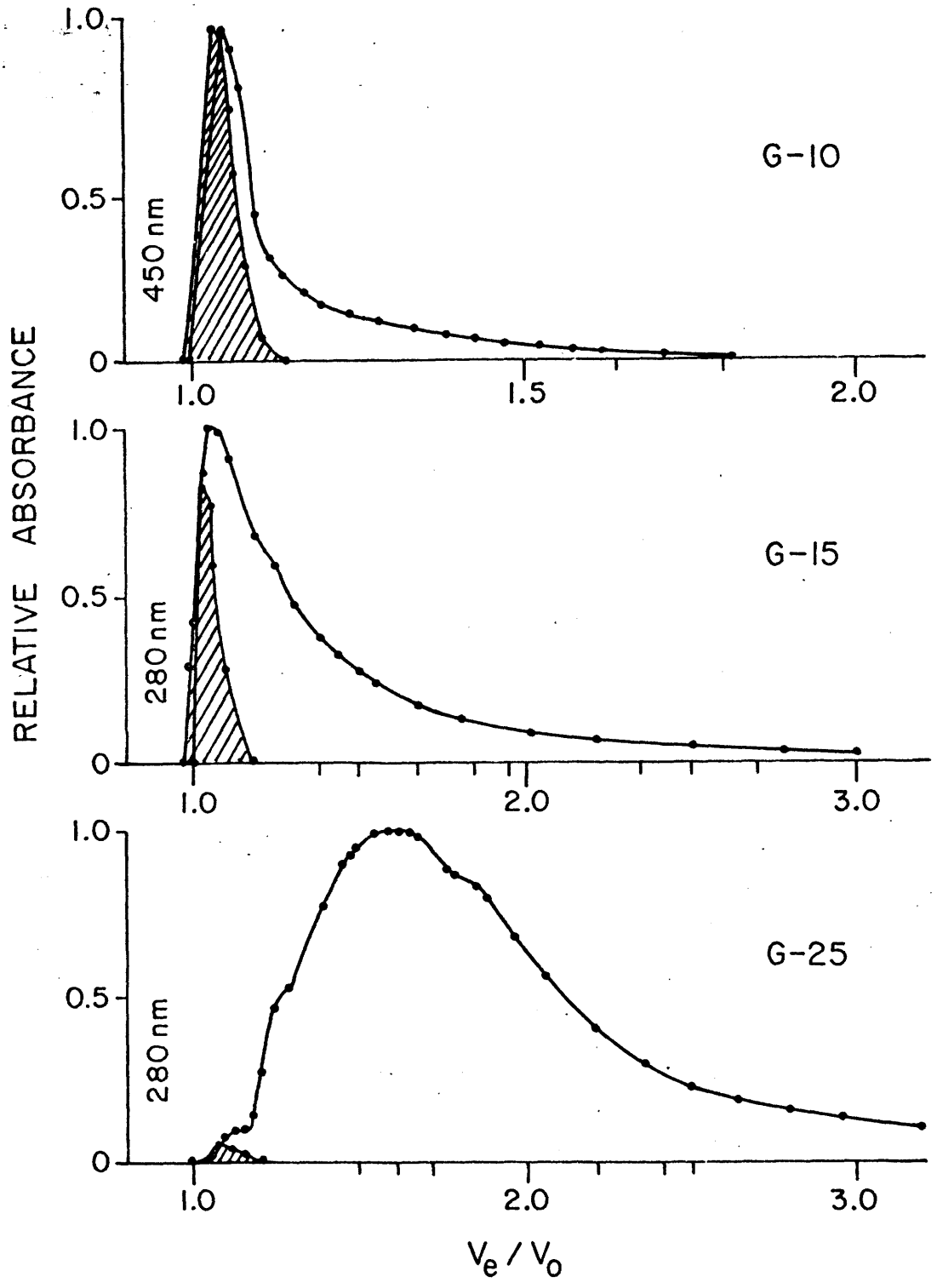
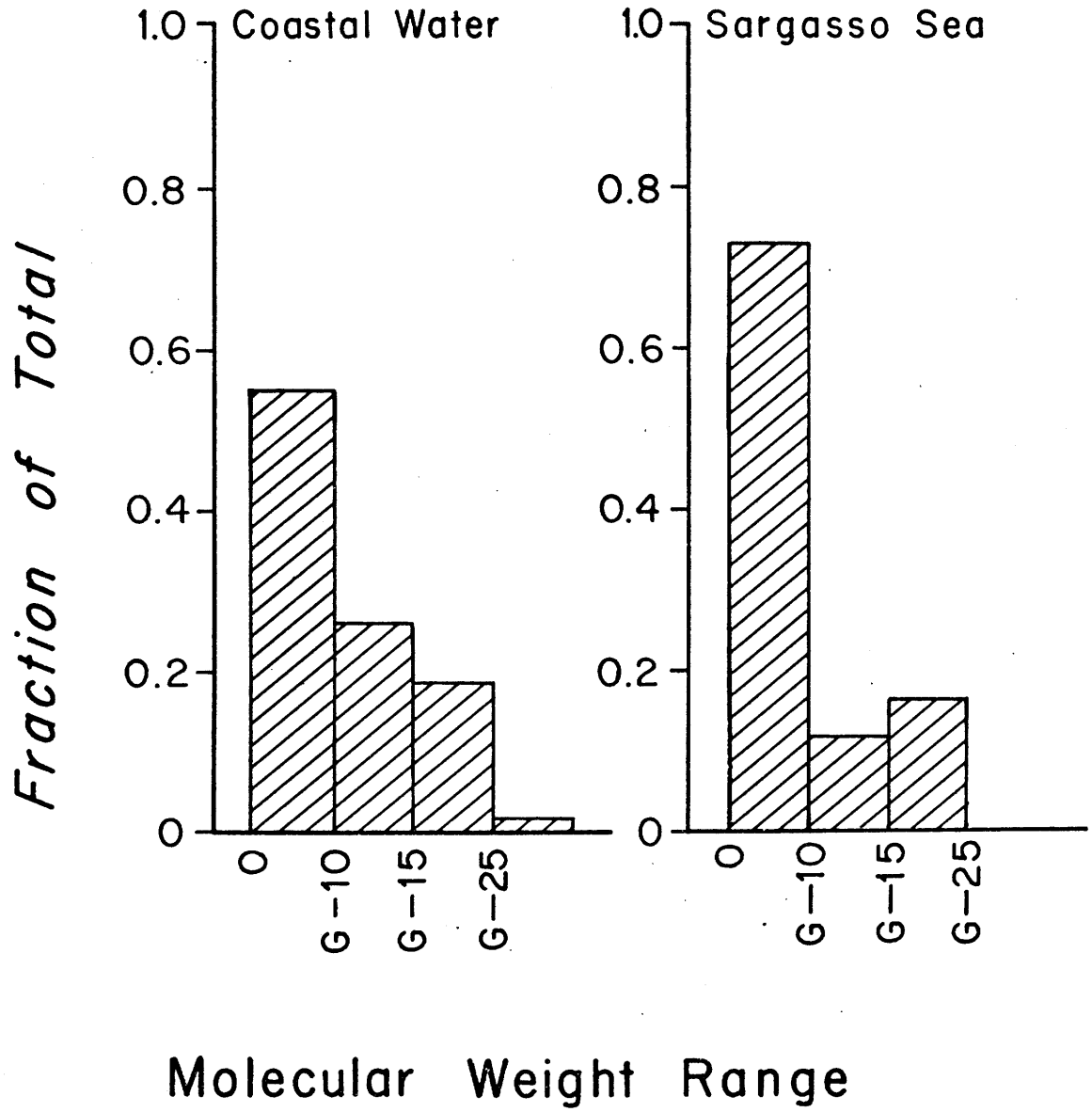

RELATIVE ABSORBANCE
1.0
0.5
0
450 nm
G-10
1.0
1.5
2.0
280 nm
G-15
3.0
G-25
V_e / V_o

Figure 1-9. Molecular weight distributions of seawater fulvic acid. Molecular weight intervals are given in terms of Sephadex gel grades.


Coastal Water
Sargasso Sea
1.0
0.8
0.6
0.4
0.2
0
Fraction of Total
0
G-10
G-15
G-25
Molecular Weight Range

CHAPTER 2

Introduction

This chapter presents carbon-13 (^{13}C) and proton (^{1}H) nuclear magnetic resonance (nmr) spectra, infrared absorption spectra, and elemental composition data on Sargasso Sea surface water fulvic acid. The data are compared to existing data and ^{13}C nmr spectra obtained on terrestrial fulvic acid and interpreted to provide structural comparison between terrestrial and seawater fulvic acids.

Carbon-13 nmr has been a useful tool for obtaining structural information on small as well as on complex organic molecules (for reviews: Stothers, 1972; Levy and Nelson, 1972; Gray and Smith, 1973; Payne, 1974). In principle, ^{13}C nmr is analogous to ^{1}H nmr (Becker, 1969; Emsley et al., 1965; Stothers, 1972; Levy and Nelson, 1972). Thus the resonance frequencies of ^{13}C nuclei placed in an external magnetic field depend on the strength of the applied magnetic field and on the local magnetic environment of each carbon atom. The advantage of ^{13}C nmr over ^{1}H nmr is that <u>every</u> carbon atom can, in principle, be observed including fully substituted aliphatic, substituted aromatic, carbonyl, acetylenic and nitrile carbons. Furthermore, the chemical shift range of ^{13}C nmr extends well over 225 ppm compared to the 15 to 20 ppm range typically encountered with ^{1}H nmr. Another advantage of ^{13}C nmr over ^{1}H nmr is that different non-interfering solvents can be used; for example, water interferes with ^{1}H nmr but not in ^{13}C nmr. This is important in this study since fulvic acid is water soluble. Because the natural abundance of ^{13}C relative to ^{12}C is only 1.1%, ^{13}C-^{13}C spin-spin interactions do not contribute significantly to the complexity

of the spectra. The low abundance does, however, reduce the sensitivity of the method. Nevertheless, Fourier transform techniques (Ernst and Anderson, 1966; and Fararr and Becker, 1971) allow spectra to be obtained in a matter of hours. Finally, the use of wide band proton decoupling not only simplifies the spectra by eliminating ^{13}C-^{1}H spin-spin interactions, but it can increase the sensitivity of the method by as much as a factor of three due to nuclear Overhauser effects (Stothers, 1972; Doddrell et al., 1972).

Experimental

The marine fulvic acid sample was isolated from seawater collected in the northwestern Sargasso Sea at stations listed in Table 2-1. The method used for isolation is reported in detail elsewhere (Chapter 1). Briefly, organic matter is adsorbed from seawater at pH 2 on Amberlite XAD-2 resin (Rohm and Haas, Philadelphia, Pa; Riley and Taylor, 1969). The resin is rinsed with distilled water to remove salt, and humic substances are eluted with 0.5 N NH_4OH. Following lyophilization, fulvic acid is dissolved in 0.01 N HCl, liquid-liquid extracted with CH_2Cl_2 to remove lipids, and recovered from the aqueous solution by lyophilization.

TABLE 2-1

Station Data - 279 mg of fulvic acid was isolated from the combined surface water from the stations listed.

Station Location	Volume (liters)	Salinity (^{o}/oo)	Temperature (^{o}C)
35^{o}13.8'N; 63^{o}35.9'W	400	36.006	27.1
33^{o}39.0'N; 62^{o}20.4'W	400	36.330	27.1
32^{o}23.0'N; 59^{o}50.0'W	800	36.591	27.4
32^{o}18.5'N; 62^{o}59.4'W	400	36.258	27.8
	2000 Total		

The soil fulvic acid was isolated from the B_h horizon of a podzol (Buckman & Brady, 1960) in Falmouth, Massachusetts, USA. The sample was extracted with 0.4 N NaOH under a nitrogen atmosphere for 24 hours. The extract was acidified to pH 2.0 and filtered through Reeve Angel Grade 934 AH glass fiber filter. The filtrate (pH 2; 0.4 M NaCl) was passed through Amberlite XAD-2 resin and fulvic acid was recovered from the resin by the same procedure used for the seawater sample.

Seawater fulvic acid (49.8 mg) was suspended in dry methanol and methylated with diazomethane in CH_2Cl_2 for 30 minutes. After drying under a stream of nitrogen gas, the methylene chloride soluble products were recovered and the residual material was again treated with diazomethane. The methylation of the residual material was repeated twice yielding 51% of the fulvic acid (25.3 mg) as CH_2Cl_2-soluble material.

Analyses for carbon, hydrogen, nitrogen and ash were carried out by Galbraith Laboratories, Inc. (Knoxville, Tenn.). Oxygen was calculated by difference.

Infrared (ir) spectra were obtained on KBr micropellets with a Perkin Elmer Model 337 grating infrared spectrophotometer with beam condensor.

Continuous wave ^1H nmr spectra were obtained at 35°C with a Hitachi-Perkin Elmer R-20B high resolution 60 MHz nmr instrument (seawater fulvic acid; 100 mg/ml; D_2O solvent; methanol internal standard) and a Varian A-60 nmr instrument [methylated seawater fulvic acid; 60 mg/ml; CD_2Cl_2 solvent; tetramethylsilane (TMS) internal standard]. Resonances are reported in parts per million (ppm) downfield from TMS.

The ^{13}C nmr spectra were obtained on a Bruker HFX-90-18 nmr spectrometer interfaced with a Digilab FTS/nmr-3 computer. This spectrometer has been elegantly modified to achieve extensive multinuclei capability (Traficante, Simms and Mulcay, 1974). It was operated in the Fourier transform mode with a fluorine field/frequency lock and full proton decoupling (1.2 KHz bandwidth); both ^{13}C nmr spectra were obtained using a 90^{o} pulse with a total pulse delay and acquisition time of 0.74 seconds. A total of 113,789 and 88,307 pulses were required for the marine and terrestrial fulvic acid, respectively. A saturated solution of KF in doubly distilled water was used to provide an inorganic source of fluoride which would not interfere with the ^{13}C nmr spectra of the fulvic acids. When the more commonly preferred hexafluorobenzene was used for the fluorine field/frequency lock signal, the aromatic region of the resulting ^{13}C nmr spectra of seawater fulvic acid was masked due to large interfering peaks at 145 and 133 ppm (the 139 ppm resonance from the carbon in hexafluorobenzene is split into a doublet due to spin-spin coupling with ^{19}F J_{CF} = 268 Hz). A 10 mm (od) coaxial nmr tube with an internal tube of 5 mm (od) diameter was used for the ^{13}C nmr experiments. The fulvic acid samples were placed in the cavity between the tubes, and the KF lock solution (containing 2% dioxane internal standard) was placed in the internal tube. The spectra were obtained at $44^{o}C$, and chemical shifts are reported in ppm downfield from TMS.

Immediately before each ^{13}C nmr study, the fulvic acid sample was dissolved in 0.01 N HCl and centrifuged (12,000 rpm, 20 min., $0^{o}C$) to remove any suspended material. A 2.5 ml solution of 48 mg/ml was used for the seawater

fulvic acid of molecular weight range 200 to 5000 with 81% less than 1500 (Chapter 1). A 3.0 ml solution of 44 mg/ml was used for the terrestrial fulvic acid. A molecular weight range of 175 to 3,600 was determined for a podzol fulvic acid from the B_h horizon by Schnitzer and Skinner (1968); a molecular weight range of 500 to 10,000 was indicated for a B_h horizon podzol fulvic acid by Levesque (1972). The soil fulvic acid used in this study is assumed to have a molecular weight range between 175 and 10,000.

Results

The isolation procedure may fractionate the seawater humic substances (Chapter 1), therefore, the terrestrial humic substances were subjected to the same procedure to obtain a comparable sample.

The *elemental compositions* and ash contents of the seawater and terrestrial fulvic acid samples are presented in Table 2-2. The major differences in elemental composition between the seawater and terrestrial fulvic acids are the relatively lower oxygen content and higher nitrogen content of the seawater sample. In addition, the H/C atomic ratio for the seawater fulvic acid is 1.61 compared to 1.15 for the terrestrial sample. Both samples have similar ash contents.

The *^{13}C nmr spectra* of the seawater and terrestrial fulvic acid samples are presented in Figure 2-1 and 2-2, respectively. The broad resonances between 170 and 185 ppm in the ^{13}C nmr spectra of both the seawater and terrestrial fulvic acid samples are characteristic of ester, carboxylic acid and amide groups. It should be noted, however, that in the terrestrial fulvic

TABLE 2-2

Elemental Composition (Ash Free Basis)

Sample Source	%C	%H	%N	%O (by difference)	% Ash	H/C Ratio
Seawater	49.98	6.76	6.40	36.86	3.37	1.61
Terrestrial	46.69	4.51	0.50	44.29	2.35	1.15

acid spectrum the carbonyl peaks are broadened toward 170 ppm.

The broad resonances in the 60-100 ppm range of both the seawater and terrestrial spectra are characteristic of polyhydroxyl groups. The sharp resonance at 67 ppm in both spectra is from the internal standard, dioxane.

Significant differences exist between the seawater and terrestrial fulvic acid spectra. The seawater fulvic acid spectrum shows more predominant resonances in the 10-60 ppm region, characteristic of aliphatic moieties. Resonances in the 110-160 ppm region of the spectra characteristic of aromatic constituents are slightly more predominant in the terrestrial fulvic acid spectrum.

The *^1H nmr spectra* of the seawater fulvic acid and of the methylated seawater fulvic acid are shown in Figure 2-3 and 2-4, respectively. The fulvic acid nmr spectrum shows broad resonances in the 1.0 to 1.7 ppm range characteristic of aliphatic protons, in the 1.7 to 2.5 ppm range characteristic of protons on carbons adjacent to functional groups such as carbonyls, aromatics or double bonds, and in the 7.3 to 7.9 ppm region characteristic of aromatic protons. The relative areas of these resonances are 15:10:1, respectively. The large peak at 5.2 ppm is due to HDO in the solvent from hydrogen exchange. The methanol internal standard is observed at 3.45 ppm.

The ^1H nmr spectrum of the methylated fulvic acid shows similar characteristics in the 1.0 to 2.5 ppm region. However, a new resonance is observed between 3.4 and 3.9 ppm due to methoxy protons in methyl esters. The minor aromatic resonance is not observed in this spectrum perhaps because of the

smaller sample size or the lower resolution of the Varian instrument. The resonance at 5.7 ppm is from a small amount of $CHDCl_2$ in the solvent. The area ratio of the resonances between 1.0 and 2.5 ppm and between 3.4 and 3.9 ppm is 4.8 to 1.0.

The *ir spectra* (Figure 2-5) demonstrate that methylation of the marine fulvic acid results in a shift from 1700 cm^{-1} to 1730 cm^{-1} in the carbonyl absorption and a reduction and sharpening of O-H stretching absorption (3000 to 3600 cm^{-1}).

Discussion

It was predicted that differential saturation of specific carbons would not be a problem using a 90^{o} pulse with a total pulse delay and acquisition time of 0.74 seconds from the molecular weight range of the fulvic acid samples and ^{13}C nmr studies of other macromolecules with similar molecular weights [specifically cholesterol (Allerhand Doddrell, and Komoroski, 1971) and low molecular weight polypeptides (Allerhand, and Komoroski, 1973; Deslauriers, Smith, and Walter, 1974; Keim, Vigna, Marshall, and Gurd, 1973; Allerhand and Oldfield, 1973) and proteins (Allerhand, Doddrell, Glushko, Cochran, Wenkert, Lawson, and Gurd, 1971; Glushko, Lawson, and Gurd, 1972; Allerhand, Childers, and Oldfield, 1973; Hunkapiller, Smallcombe, Whitaker, and Richards, 1973)]. Test spectra of 0.1 M cholesterol in d_6-benzene, obtained using the same instrumental conditions, support this prediction. Furthermore, compared to protonated or aliphatic carbons, the carbonyl carbons in most macromolecules have

relatively longer spin-lattice relaxation times and are therefore more subject to saturation (Allerhand and Komoroski, 1973). The observation of the predominant carbonyl resonances in the fulvic acid spectra demonstrates that saturation is not a significant problem in these studies, also in agreement with the above prediction.

Nuclear Overhauser effects can complicate interpretation of ^{13}C nmr spectra, particularly if comparisons between peak areas in a given sample are to be made (Stothers, 1972). Specifically, actual aliphatic:aromatic carbon ratios in the individual fulvic acid samples cannot be accurately determined by simply comparing the aliphatic (10-60 ppm) and aromatic (110-160 ppm) peak areas. However, considering the molecular weight ranges of the seawater and terrestrial fulvic acid samples, it is reasonable to suggest that similar nuclear Overhauser effects are occurring in both samples (Doddrell, Glushko, and Allerhand, 1972; Allerhand and Oldfield, 1973). Thus, one can compare the relative peak areas between the spectra of seawater and terrestrial fulvic acid.

Several factors may contribute to the broad and poorly resolved resonances observed in the marine and terrestrial fulvic acid spectra. One is molecular weight dependent rotational correlation times (Doddrell, Glushko, and Allerhand, 1972; Allerhand and Hailstone, 1972; Becker, 1969). However, this explanation is unlikely, considering ^{13}C nmr relaxation studies on molecules with molecular weights similar to the fulvic acid samples studied here (Allerhand, Doddrell and Komoroski, 1971; Allerhand and Komoroski, 1973; Deslauriers, Smith, and

Walter, 1974; Keim, Vigna, Marshall, and Gurd, 1973; Allerhand and Oldfield, 1973).

Another factor could be line broadening from paramagnetic materials and free radicals in the samples. The presence of paramagnetic metal ions and free radicals has been demonstrated in humic materials (Steelink and Tollin, 1967), therefore, their contribution to line broadening cannot be ignored. Complexed paramagnetic metals can interact with ^{13}C nuclei causing extremely fast relaxation times and, therefore, line broadening (Stothers, 1972; Levy and Nelson, 1972; Becker, 1969).

In addition to line broadening, chemical shifts are often changed by a few to several thousand ppm when carbons are complexed with paramagnetic metal ions or when free radicals are present (Levy and Nelson, 1972; Stothers, 1972). Carbons which are shifted more than a few hundred ppm would not be observed in the 0 to 250 ppm spectral region.

Finally, the broad resonances in the aliphatic regions of both spectra, and in the spectrum of the marine fulvic acid in particular, may also simply reflect the extreme molecular complexity of the materials. This phenomenon of line broadening due to molecular complexity has been observed in other macromolecules such as proteins and nucleic acids (Payne, 1974; Lauterbur, 1970; Chien and Brandts, 1971; Freedman, Lyerla, Chacken, Cohen, 1973; Allerhand, Childers, and Oldfield, 1973; Glushko, Lawson and Gurd, 1972; Komoroski and Allerhand, 1972). However, even in proteins with molecular weights as high as 310,000 it is still possible to observe well resolved resonances from many

of the side-chain aliphatic and aromatic carbons (Payne, 1974). These peaks can be resolved in spite of slightly differing magnetic environments (due to anisotropy in the magnetic susceptiblity of neighboring atoms or groups such as phenyl rings and carbonyl groups) because of the limited number of amino acids present as repeating subunits. The presence of a complex array of carbon species may readily account for the broad resonances observed in the ^{13}C nmr spectra, although the presence of paramagnetic metals and free radicals may be a contributing factor.

The ^{13}C nmr spectrum of seawater fulvic acid shows a much greater abundance of resonances from aliphatic carbons than are present in the soil fulvic acid spectrum. Conversely, the aromatic resonances are more prominent in the soil fulvic acid spectrum than in the seawater spectrum. This suggests major structural differences between the fulvic acids from the two environments. The more aliphatic character of the seawater fulvic acid is consistent with the higher H/C ratio (Table 2-2). The main features of the ^{1}H nmr spectra of seawater fulvic acid (Figure 2-3 and 2-4) are the large broad aliphatic resonances between 1.0 and 2.5 ppm and the lack of large aromatic resonances between 7.0 and 9.0. These ^{1}H nmr data are in support of the highly aliphatic character of seawater fulvic acid indicated by the ^{13}C nmr data.

The less aromatic character of sedimentary humic substances of lacustrine and marine origin as compared to terrestrial humic substances has been described previously. Differences in density, elemental composition and the results of chemical studies led Ishiwatari (1971) to assign a less aromatic character to

humic substances isolated from lake sediments. Analyses of functional groups in marine sedimentary fulvic acid demonstrate a lower phenol content than is present in terrestrial fulvic acid (Rashid and King, 1972); this also suggests a less aromatic character for marine humic substances.

The less aromatic, more aliphatic character of the seawater fulvic acid may reflect the lack of abundant aromatic precursors in the marine environment. Lignin is believed to be an important component in the formation of soil humic substances (Hurst and Burges, 1967). It is not abundant in marine plants except for some benthic and marsh grasses (Moore, 1969; Leo and Barghoorn, 1970; Gardner and Menzel, 1974).

The abundance of carbonyl oxygen in both fulvic acid samples is demonstrated by the ^{13}C nmr resonances between 170 and 185 ppm. Differentiation between carboxylic acid, ester and amide functional groups is not possible from the ^{13}C nmr data; however, resonances characteristic of aldehydes and ketones are sufficiently separated (20 to 30 ppm downfield from other carbonyls) to allow their distinction. It is noteworthy that this distinction cannot be made from the fulvic acid ir data because of the broad carbonyl absorbances. The lack of significant resonances in the 190 to 210 ppm region in both ^{13}C nmr spectra indicate that the major carbonyl groups in the fulvic acid samples are acids, esters and amides. The broader carbonyl resonance extending toward 170 ppm in the terrestrial sample suggests the presence of a greater variety of carbonyl groups here than in the seawater sample.

Carboxylic acid groups in the seawater fulvic acid are indicated by the

shift in the ir carbonyl absorbance from 1700 cm^{-1} to 1730 cm^{-1} upon methylation of the sample. The accompanying decrease and sharpening of the OH stretching absorbance (3000 to 3600 cm^{-1}) results from the formation of methoxyl groups from the OH groups of acids and perhaps phenols. In addition, the appearance of the new ^{1}H nmr resonance at 3.4 to 3.9 ppm upon methylation of the fulvic acid is also evidence of the formation of methyl esters from carboxylic acids.

Absorption in the 60 to 100 ppm region of the ^{13}C nmr spectra of both fulvic acid samples suggests the presence of polyhydroxyl groups such as sugars. This observation is consistent with the intense infrared absorption in the 3000 to 3500 cm^{-1} region characteristic of O-H stretching. That absorption persists after methylation indicates the presence of alcoholic functions. The highly aliphatic character and high oxygen content (from elemental analysis) require that there be highly oxygenated units within the fulvic acid structure. Sugar-like moieties are consistent with these spectral observations. The humic substances in seawater must be formed from the precursors present such as amino acids, carbohydrates, lipids and pigments. The formation of humic substances by processes analogous to the browning reaction has been suggested by several investigators (Kalle, 1966; Nissenbaum, 1974; Jackson, 1975). The incorporation of marine lipids and pigments into the products of the browning reaction could account for the structural features indicated by the present work.

Summary

The results of this chapter are summarized in Table 2-3.

TABLE 2-3

Summary of Results

Seawater Fulvic Acid Structural Feature	Method ^{13}C nmr	^{1}H nmr	ir	El. Comp.
Complex array of aliphatic carbons	10-60 ppm strong broad resonances	1.0-2.5 ppm strong broad resonances	--	H/C = 1.61
Polyhydroxy groups	60-100 ppm strong broad resonances	--	Persistence of 3000-3600 cm^{-1} after methylation	High oxygen low eq. wt.
Low aromatic character	110-160 ppm weak resonances	7.0-9.0 ppm weak resonances	--	H/C = 1.61
Amide, acid ester functionality	170-185 ppm strong resonances	--	a) Carbonyl bands at 1700 and 1560 cm^{-1} b) Shift to 1730 cm^{-1} upon methylation	High oxygen
Low abundance of ketones and aldehydes	190-210 ppm no resonances	--	--	--

Figure 2-1. Carbon-13 nmr spectrum of Sargasso Sea surface water fulvic acid.

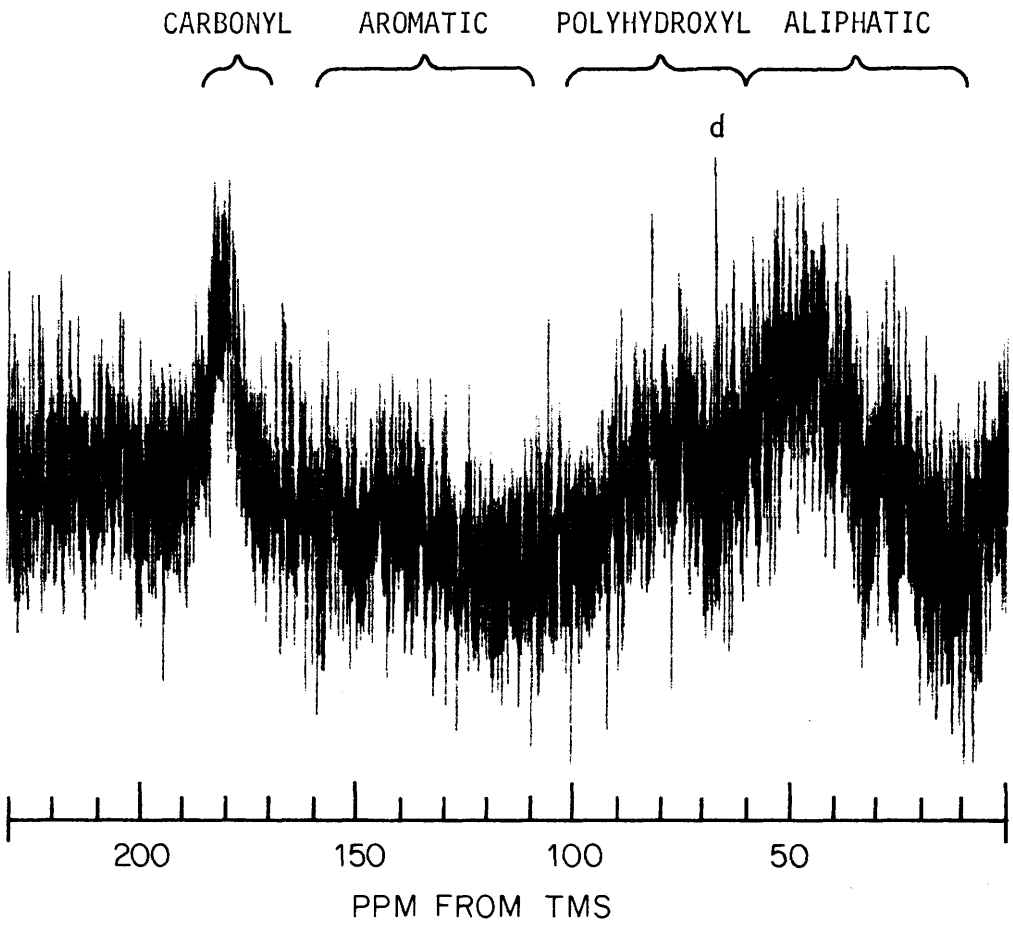

CARBONYL
AROMATIC
POLYHYDROXYL
ALIPHATIC
d
200
150
100
50
PPM FROM TMS

Figure 2-2. Carbon-13 nmr spectrum of terrestrial fulvic acid.

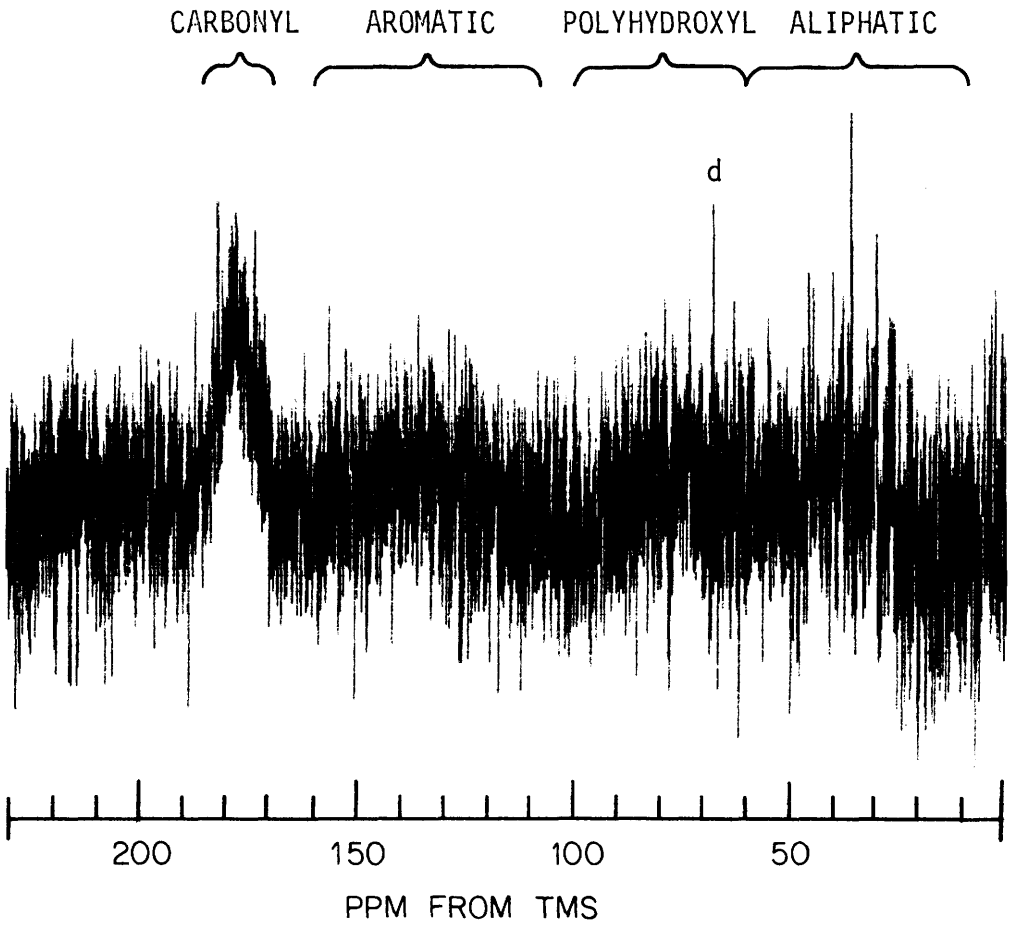

CARBONYL
AROMATIC
POLYHYDROXYL
ALIPHATIC
d
200
150
100
50
PPM FROM TMS

Figure 2-3. Proton nmr of Sargasso Sea surface water fulvic acid.

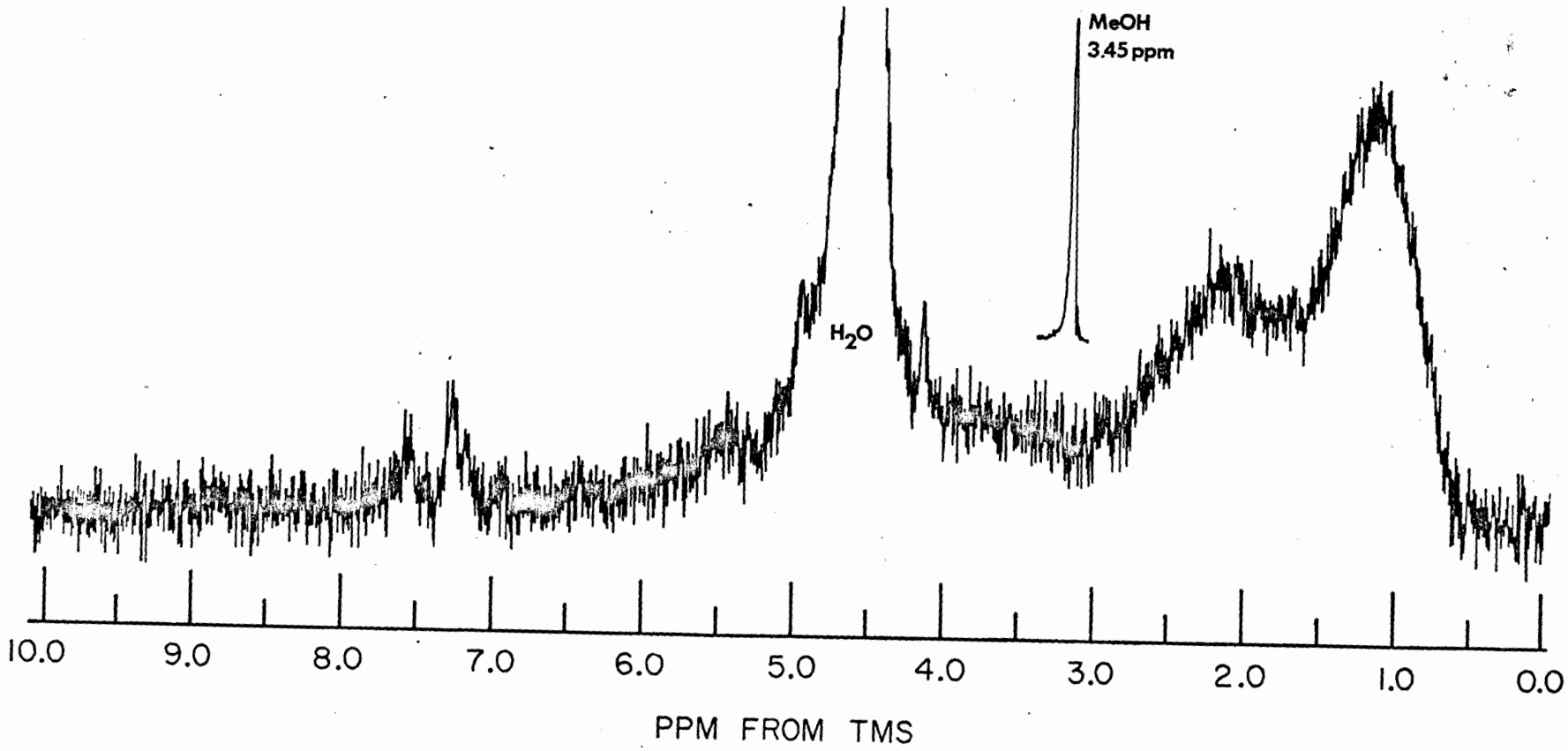

MeOH
3.45 ppm
H_2O
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
PPM FROM TMS

Figure 2-4. Proton nmr of CH_2Cl_2-soluble methylated Sargasso Sea surface water fulvic acid.

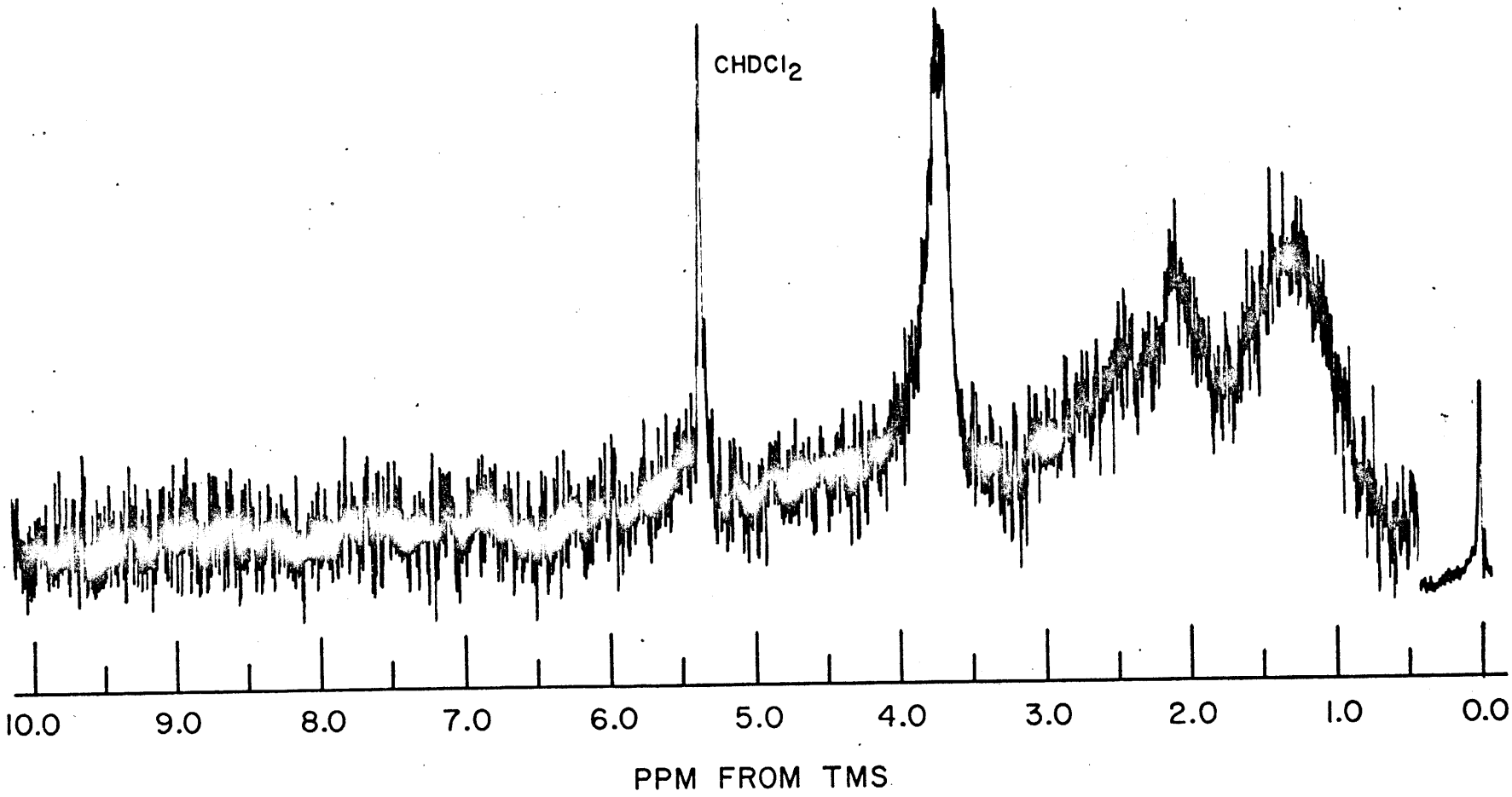
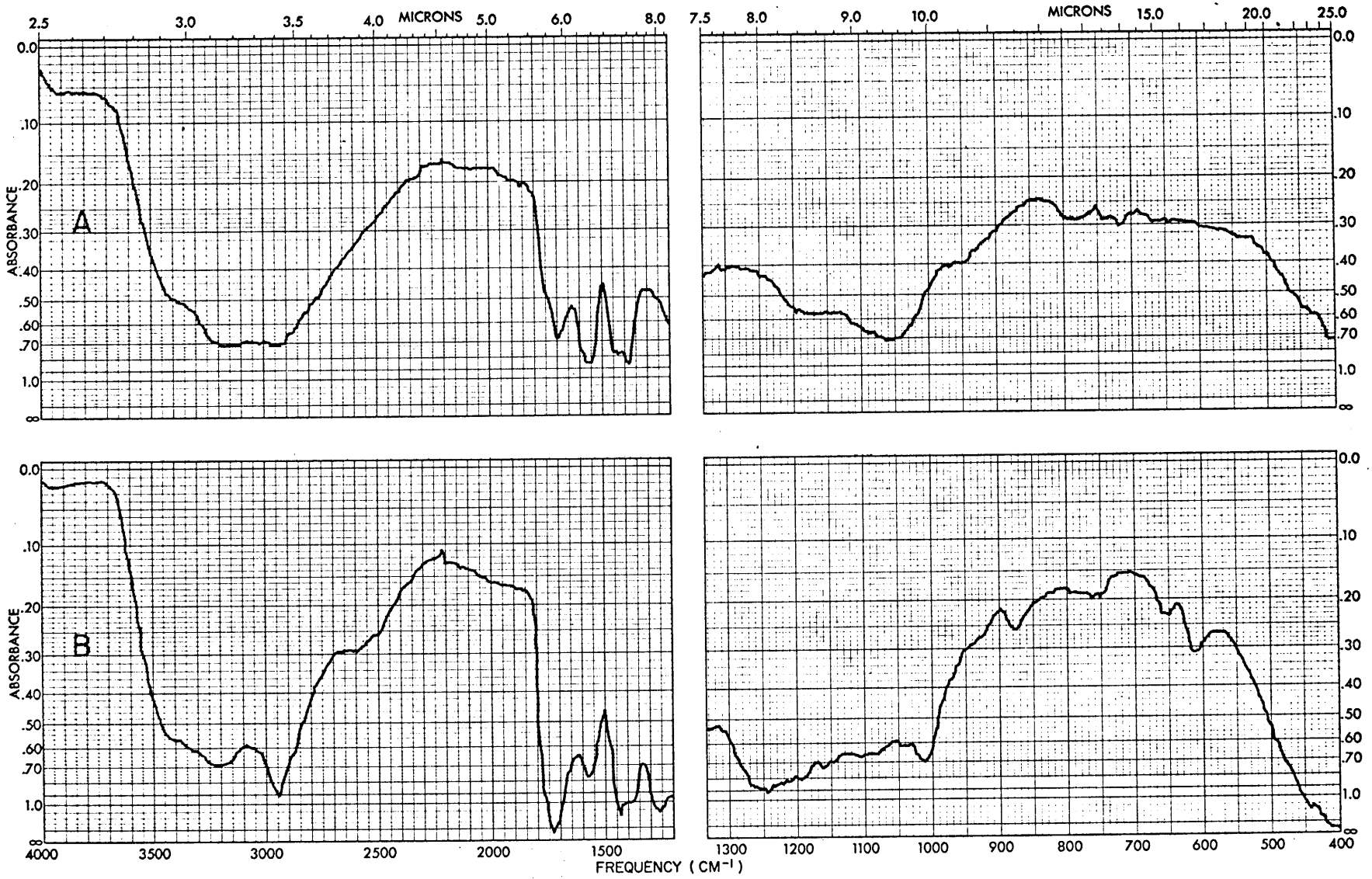

CHDCl2
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
PPM FROM TMS

Figure 2-5. Infrared spectra of Sargasso Sea surface water fulvic acid (A) and CH_2Cl_2-soluble methylated Sargasso Sea surface water fulvic acid (B).


2.5
3.0
3.5
4.0
MICRONS
5.0
6.0
8.0
7.5
8.0
9.0
10.0
MICRONS
15.0
20.0
25.0
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
ABSORBANCE
A
B
4000
3500
3000
2500
2000
1500
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM⁻¹)

CHAPTER 3

Introduction

This chapter presents the results of chemical studies of Sargasso Sea and coastal water fulvic acid and their interpretation in terms of the structural features of marine humic substances.

The rationale in these studies was first to investigate the intact fulvic acid and then proceed to studies of fulvic acid components freed by hydrolysis. Finally, drastic chemical reduction was used to simplify the structure for identification of carbon skeletal features. Because of the limited sample size, techniques were used to gain maximum information from the amount of sample expended.

Experimental

General Equipment and Reagents

Infrared (ir) spectra were obtained on a Perkin Elmer Model 337 grating infrared spectrophotometer with KBr pellets or NaCl plates.

Varian model 1200 or model 1400 gas chromatographs equipped for on-column injection (injector temperature 180°C to 210°C) and with flame ionization detectors (detector temperture 300°C) were used.

Solvents were distilled in all-glass stills before use. Ether was distilled from Na metal. Cyclohexane was washed with conc. H_2SO_4, dried (Na_2SO_4) and then distilled from Na metal. Water was distilled from basic $KMnO_4$ under precombusted (800°C; CuO) nitrogen gas.

Reagent grade chemicals were used. NaOH was obtained by evaporation of aqueous sodium methoxide, prepared from reagent grade Na metal (Matheson, Cole,

and Bell, Norwood, Ohio) and methanol.

Diazomethane was prepared from N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chemical Company, Milwaukee, Wisconsin) by the method of Fales et al. (1973).

Samples: Fulvic acid was isolated from Sargasso Sea surface waters and coastal waters near Woods Hole, Massachusetts, USA. Sample locations and the isolation procedure are described in Chapter 1.

The low molecular weight fraction of the Sargasso Sea fulvic acid retained by Sephadex G-10 gel (Chapter 1) was recovered by lyophilization.

Blank of Isolation Procedure: A blank of the fulvic acid isolation procedure (Chapter 1) was carried out beginning with the NH_4OH elution of 250 cc of Amberlite XAD-2 resin. A white solid weighing 0.7 mg was recovered and analyzed by ir spectroscopy.

High Resolution Mass Spectrometry: High resolution mass spectra (70 ev) of the volatiles from Sargasso Sea fulvic acid were obtained on a CEC-21-110-B high resolution mass spectrometer at Massachusetts Institute of Technology, during temperature programming of the direct insertion probe from 50 to 425°C. The instrument was calibrated with perfluoroalkanes and the data were processed with an IBM 1800 computer.

Reflux Extraction of Fulvic Acid: Sargasso Sea fulvic acid (50 mg) was refluxed in 5 ml methylene chloride (CH_2Cl_2) for 10 hours. The suspension was cooled and centrifuged (1800 g; 15 min.). The CH_2Cl_2 solution was concentrated under a stream of nitrogen and the residue was weighed. It was then methylated with diazomethane and analyzed by GC on a Varian 1200 gas chromatograph (10%

FFAP; Chromosorb W HP; 5 ft.; 2 mm i.d. glass column; temperature programmed from 130 to 230°C at 4°C/min.). The sensitivity limit was 50 ng/component.

Methylated Fulvic Acid: Methylation of the total and low molecular weight fraction of Sargasso Sea fulvic acid with diazomethane (Chapter 2) yielded 51% and 97%, respectively, of methylene chloride-soluble material.

The methylated fulvic acid samples were analyzed by combined gas chromatography-mass spectrometry (GC-MS) with a Perkin Elmer 900 gas chromatograph (2% OV-17; Chromosorb W HP; 6 ft.; 1/8" steel column; temperature programmed; 80°C to 365°C at 12°C/min.) coupled to a Hitachi-Perkin Elmer RMU-6L mass spectrometer and an IBM 1800 computer. The sample was also analyzed after silylation using N,O-bis (trimethylsilyl)trifluoroacetamide.

Thin-Layer Chromatography (TLC): The methylated fulvic acid samples were chromatographed in methanol:benzene (1:3) on Quanta/Gram Q5 silica thin layer plates (Quantum Industries, N. J.). Chromatograms were developed with short-wave uv light and with iodine vapor. Four fractions were recovered by scraping and eluting the silica gel with methanol in a sintered-glass funnel. Four fractions were collected (Figure 3-6). Fraction 1 (least polar) was analyzed by gas chromatography (2% Apiezon L on Chromosorb W HP; 6 ft.; 1/8" steel column; temperature programmed from 60°C to 280°C at 6°C/min.). All four fractions were analyzed by probe mass spectrometry using a Finnigan model 1015C quadrapole mass spectrometer. The probe was heated from 30°C to over 200°C while accumulating one mass spectrum every seven seconds. TLC blanks corresponding to these four fractions were also analyzed.

Hydrolysis of Fulvic Acid: Sargasso Sea surface water fulvic acid was hydrolyzed with 6 N HCl at 100°C for 22 hours under nitrogen. A blank starting with 6 N HCl was carried through the entire procedure.

Amino Acids in Hydrolyzate: The hydrolysis products were diluted with distilled water and applied to a 1 cm x 10 cm column of Bio-Rad Analytical grade AG 50W - X8 cation exchange resin, 100-200 mesh (Bio-Rad Laboratories, Richmond, California), cleaned before use by extensive acid and base washing. The column was eluted with two bed volumes of distilled water and then eight bed volumes of 1 N NH_4OH. The elution was monitored with Ninhydrin tests. The base eluent containing the amino acids was concentrated at 35°C on a rotary evaporator and was transferred to a 1 ml reactor vial and dried under a stream of nitrogen gas. The dried sample, a blank and a standard amino acid solution were treated with 3 N HCl/n-butanol solution at 90°C for one hour. The solutions were dried under nitrogen and treated with excess trifluoroacetic acid anhydride in methylene chloride at 3°C for 16 hours. These products were then dried in a vial under nitrogen, a known volume of methylene chloride containing n-butyl stearate (internal standard) was added through a septum cap before on-column injection into a Varian model 1400 gas chromatograph [Tabsorb (Regis Chem. Co., Morton Grove, Ill.); 6 ft. 2mm I.D. glass column; temperature programmed from 75°C to 210°C at 8°C/min.]. Amino acid concentrations were determined by comparing peak areas to the peak area of the internal standard and using the response factors from the standard amino acids. The identification of the amino acids was confirmed by GC-MS under the conditions described above.

Methylene Chloride-Soluble Products in the Hydrolyzate: Four fractions (acids, phenols, neutrals, bases) of CH_2Cl_2-soluble material were obtained from the total hydrolyzate by the liquid-liquid extraction scheme in Figure 3-1. These materials represent 18% by weight of the original fulvic acid. Each fraction was analyzed by GC under the conditions of Table 3-1 and by GC-MS with a Finnigan model 1015C GC-MS system. Also, mass spectra were obtained while heating the probe from 30 to 250°C.

Exhaustive Reduction of Fulvic Acid (Figure 3-2): Fulvic acid (50 mg) was methylated as described in Chapter 2. A methanol solution of the methylated fulvic acid was hydrogenated at 5,200 psi H_2 and 180°C for 36 hours over 36 mg copper barium chromium oxide (Cu-Ba Chromite). The catalyst was prepared according to Lazier and Arnold (1943) and activated immediately before use at 3500 psi H_2 and 100°C for 1.5 hours. After hydrogenation, the sample and catalyst were filtered through an extracted Soxhlet thimble and the catalyst was Soxhlet extracted with refluxing methanol for 16 hours. The combined extract and filtrate was concentrated at 20° on a rotary evaporator and dried under a stream of nitrogen. An infrared spectrum of this material was obtained on NaCl plates.

The hydrogenation products were then brominated with 500 mg of dibromotriphenylphosphorane (Wiley et al., 1964; Schaefer and Weinberg, 1965a; Schaefer and Weinberg, 1965b; Horner et al., 1959) in 5 ml of freshly distilled acetonitrile at 105° for 24 hours under nitrogen in a sealed tube. The reaction products were diluted with 75 ml of water and liquid-liquid extracted with freshly distilled diethyl ether. The ether was dried over Na_2SO_4 and evaporated to dryness

TABLE 3-1

Gas Chromatography Conditions for CH_2Cl_2-Soluble Hydrolysis Products (Figure 3-1)

Fraction	Derivatization (Reagent)	Column and Temperature
Acids	a) methylation (CH_2N_2)	a) 2% SE-30/Chromosorb W HP 5 ft. Glass Column 75-250°C at 8°C/min.
		b) 2% Apl**/Chromosorb W HP 5 ft. Glass Column 75-280°C at 8°C/min.
		c) 10% FFAP/Chromosorb W HP 5 ft. Glass Column 130-230°C at 4°C/min.
	b) silylation (BSA/TMCS)*	2% Apl**/Chromosorb W HP 5 ft. Glass Column 75-280°C at 8°C/min.
Phenols	a) methylation (CH_2N_2)	a) 2% SE-30/Chromosorb W HP 5 ft. Glass Column 75-250°C at 8°C/min.
		b) 10% FFAP/Chromosorb W HP 5 ft. Glass Column 130-230°C at 4°C/min.
	b) silylation (BSA/TMCS)*	2% Apl**/Chromosorb W HP 5 ft. Glass Column 75-280°C at 8°C/min.
Neutral	None	2% SE-30/Chromosorb W HP 5 ft. Glass Column 75-250°C at 8°C/min.
Bases	Trifluoroacetylation (TFAA)***	50' DEGS SCOT (GC-MS) 80°-180°C at 4°C/min.

*N,O-bis(trimethylsilyl)acetamide/Trimethylchlorosilane: 10/1.

**Purified Apiezon L - hexane eluent from alumina.

***Trifluoroacetic anhydride - 30% in CH_2Cl_2.

at 15°C on a rotary evaporator. The residue was taken up in a small volume of freshly distilled methanol.

The bromination products were reduced for 10 hours with stirring over one gram 10% $Pd/BaCO_3$ (Mozingo, 1955) at 1 atm. H_2 and 30°C, in 2% KOH/methanol. The solution and catalyst were filtered through an extracted Soxhlet thimble and the catalyst was Soxhlet extracted for 12 hours with refluxing methanol. The combined extract and filtrate were diluted with water and liquid-liquid extracted with freshly distilled cyclohexane. The cyclohexane was dried over anhydrous Na_2SO_4 and concentrated to a small volume at 30°C on a rotary evaporator. This solution was applied to a 1 x 16 cm alumina (grade AL0102 P, Harshaw Chem. Co., Cleveland, Ohio; deactivated with 1.5% water) column and eluted with 5 column volumes of pentane (Fraction 1) and one column volume each of 2% CH_2Cl_2/pentane, 5% CH_2Cl_2/pentane, 10% CH_2Cl_2/pentane and 20% CH_2Cl_2/pentane (Fractions 2-5). Triphenylphosphine oxide (product of brominating reagent) was eluted with 100% CH_2Cl_2.

Fractions 1 through 5 were analyzed by GC (2% Apiezon L on Chromosorb W HP; 7 ft., 1/8" steel column; temperature programmed from 75 to 280°C at 6°C/min.). Any fraction showing peaks in the gas chromatogram was further analyzed by GC-MS on a Finnigan model 1015C GC-MS under the same GC conditions and at 55 eV ionizing voltage; some spectra were recorded at 12 eV ionizing voltage for the preferential detection of aromatic hydrocarbons.

Fraction 1 of the coastal water fulvic acid reduction products was further chromatographed on silica gel (deactivated with 1.5% water) to separate saturated hydrocarbons (pentane eluent, 3 bed volumes) from aromatic hydrocarbons (CH_2Cl_2 eluent, 3 bed volumes). Both fractions were analyzed by GC and GC-MS.

A blank of the entire procedure starting with diazomethane in methanol and proceeding through the hydrogenation, bromination, hydrogenation and chromatography steps was also analyzed by GC and GC-MS.

Results

Mass Spectrometric Probe Analysis: A few of the prominent ion series in the high resolution mass spectra (HRMS) of Sargasso Sea fulvic acid are graphically superimposed on a low resolution mass spectrum (LRMS) of the sample in Figure 3-3. Peaks in the LRMS at even mass units extend beyond m/e 500, and the HRMS show that nearly every peak in the LRMS is composed of several ions of different composition. Further inspection of the HRMS data indicates that most ions of a given heteroatom composition are components of alkyl series extending to between 20 and 25 carbons. Polyoxygenated ions and nitrogen containing ions are predominant at all temperatures. Aromatic ions are present in low abundance.

Gas Chromatography-Mass Spectroscopy: The reconstructed gas chromatogram from the GC-MS analysis of the methylated/silylated fulvic acid (Figure 3-4) shows two partially resolved peaks from methyl benzoate and dimethyl phthalate (contaminants from isolation procedure, Chapter 1) superimposed on the large unresolved envelope beginning at 200°C and extending beyond the end of the temp-

erature program (365°C). All mass spectra within this unresolved envelope have ions at every mass (Figure 3-5) and attempts at interpretation were fruitless.

Reflux Extraction: The gas chromatogram of CH_2Cl_2 soluble material obtained by reflux extraction showed only one peak. Co-injection demonstrated the retention time to coincide with methybenzoate, a contaminant from the isolation procedure (Chapter 1).

Thin-Layer Chromatography: The thin-layer chromatograms of the total and low molecular weight methylated fulvic acid (Figure 3-6) are nearly identical and suggest that gel permeation chromatography has separated similar materials of different molecular size and has not fractionated the fulvic acid chemically.

The least polar fraction of a preparative TLC run (Fraction 1, Figure 3-6) was analyzed by GC. The gas chromatogram shows a large unresolved envelope above the TLC blank. GC-MS analysis of the TLC fraction 1 showed extremely complex mass spectra and mass chromatograms which followed the shape of the reconstructed gas chromatogram. Silylation of that material and GC with on-column injection on a glass column did not improve the appearance of the gas chromatogram.

Mass spectra (Figure 3-7) were taken at the maximum of the total ion current displayed during probe heating of each TLC fraction (Figure 3-6). As expected, the least polar TLC fraction is distilled from the probe at the lowest temperature (Figure 3-7).

Hydrolysis: The gas chromatograms of the amino acid derivatives from the hydrolysis of Sargasso Sea surface water fulvic acid, the corresponding blank, and the reconstructed gas chromatograms of that sample and of the standard

solution are presented in Figures 3-8 and 3-9. The amino acids account for 1.2% of the total fulvic acid nitrogen and 0.6% of the total fulvic acid by weight (Table 3-2).

GC-MS analysis of the CH_2Cl_2-soluble bases recovered after acid hydrolysis (Table 3-1) showed a series of peaks; however, mass spectra were inadequate for identification of components. Decomposition of this sample during storage at -5°C under nitrogen was indicated by the development of brown color and a broad unresolved gas chromatogram when further analysis was attempted. Further characterization of this sample was not pursued.

No methylene chloride-soluble, neutral products were observed either by weight or by GC analysis.

Gas chromatograms of the methylene chloride soluble acid and phenol fractions obtained after hydrolysis of the fulvic acid exhibited an unresolved broad envelope above the background. Mass spectra during GC-MS analysis show the complex distribution of ions characteristic of the total fulvic acid mixture. No specific interpretation of the data was possible. An example of a mass spectrum from the GC-MS of the acid fraction is given in Figure 3-10.

Reduction Experiments: The ir spectra of the methylated Sargasso fulvic acid and of the high pressure hydrogenation product (Figure 3-2) are compared in Figure 3-11. Generally, the spectrum of the reduction product is much less complex. The carbonyl band (1730 cm^{-1}; ester) is narrower and less intense. The alkyl C-H stretching absorbance (2840 to 2970 cm^{-1}) is predominant and absorbances characteristic of alkyl groups are observed in the C-H bending region (1300 to 1500 cm^{-1}).

TABLE 3-2

Results of Amino Acid Analysis

Amino Acid	Relative Mole %
Alanine	12.4
Valine	4.6
Glycine	29.8
Leucine	2.2
Proline	6.8
Threonine	6.8
Serine	16.7
Aspartic Acid	11.4
Glutamic Acid	10.1

Weight % of FA = 0.6%

% of FA Nitrogen = 1.2%

The gas chromatograms of fraction 1 of the reduction products (Figure 3-2) of the Sargasso Sea fulvic acid and blank are presented in Figure 3-12, along with the gas chromatogram of a standard mixture of even-numbered n-alkanes, C_{12} to C_{30}. The reconstructed gas chromatogram and ion plots for m/e 57; 71; 85; 99; 113 (alkyl ions) and m/e 77; 91; 105; 119; 133 (alkyl benzenes) (Figure 3-13) indicate that alkanes and alkyl benzenes account for all the peaks observed in the gas chromatogram. The presence of n-alkanes of 12 to 26 carbon chain lengths is evident from the comparison of GC retention times and mass spectral data (Figure 3-13b). Even n-alkanes predominate with the distribution maximum at $C_{18}H_{38}$. Branched alkanes, suggested by peaks of the proper retention time, occur at low levels.

The aromatic hydrocarbons (Figure 3-13c) in the Sargasso sample are present as four homologous series of alkyl benzenes of the structures indicated in Figures 3-14, 3-15, 3-16, and 3-17. Benzenes with alkyl side chains of $C_{10}H_{21}$, $C_{11}H_{23}$, and $C_{12}H_{25}$ are predominant with minor amounts of $C_{13}H_{26}$ and no C_9H_{19} side chains present for each series. The substitution patterns were assigned by following the principles of mass spectrometry of alkyl benzenes described in Grubb and Meyerson (1963) and Budzikiewicz et al. (1967), and by comparison of GC retention times with those of similar known compounds. Exact structures of the side chains is not known.

The GC-MS data for fraction 1 of the reduction products (Figure 3-2) of coastal water fulvic acid (Figure 3-18) indicate the presence of alkanes (Figure 3-18b) with a similar range and distribution as in the Sargasso sample but the

maximum at $C_{20}H_{42}$. The same twelve aromatic components described for the Sargasso sample can be discerned in the coastal sample but many other unidentified aromatic compounds are also present (Figure 3-18c).

The alkanes and aromatic compounds represent approximately 3% of the fulvic acid carbon. The ratio of saturated to aromatic hydrocarbons in these samples is approximately 20:1.

No components were observed in the alumina chromatography fractions 2, 3, or 4 of the reduction products (Figure 3-2).

Fraction 5 of the reduction products of both samples (20% CH_2Cl_2/pentane) contained an even-numbered series of straight-chain methyl esters (Figure 3-19). Structures were confirmed by comparison with GC retention times and mass spectra of standard compounds.

Discussion

Seawater fulvic acid is extremely complex; this is evident from the high resolution mass spectra (Figure 3-3), which shows ions of several different elemental compositions at every mass unit to beyond 500. Ions containing several oxygen and nitrogen atoms predominate, but saturated and unsaturated homologous series containing heteroatoms are also observed, as are aromatic series though at lower abundance. Since probe temperatures above 200°C were required, these ions may represent pyrolysis fragments of the fulvic acid.

Methylation and silylation of the fulvic acid followed by GC-MS analysis resulted in a broad unresolved chromatogram (Figure 3-4) and extremely complex mass spectra (Figure 3-5). This is further evidence for the complex composition of the fulvic

acid sample; however, some of this complexity may arise from decomposition of the sample during GC analysis.

Reflux extraction of soil fulvic acid with organic solvents has succeeded in isolating alkanes (2.4 mg/100 g^{FA}) and fatty acids (5 mg/100 g^{FA}) which were identified by ir spectroscopy and mass spectrometry after alumina chromatography (Barton and Schnitzer, 1963; Ogner and Schnitzer, 1971; Khan and Schnitzer, 1971). Increased yields (75 mg/100 g^{FA}; 47 mg/100 g^{FA}, respectively) of these components were obtained if the sample was methylated prior to extraction (Ogner and Schnitzer, 1970; Schnitzer and Ogner, 1970; Schnitzer and Khan, 1972). Hydrocarbons and fatty acids do not fit the solubility definition of fulvic acid and their presence suggests that they are retained in hydrophobic sites within the tertiary structure of the soil fulvic acid. The increased yield of hydrocarbons and fatty acids upon methylation is apparently the result of decreased hydrogen bonding through acidic OH groups within the fulvic acid; methylation loosens or "denatures" the tertiary structure and permits the extraction of non-polar molecules.

Reflux extraction of the seawater fulvic acid (50 mg) only yielded some benzoic acid (0.3 μg), a contaminant from the isolation procedure (Chapter 1). Methylation of the extracted fulvic acid and TLC analysis of the methylene chloride-soluble fraction did not reveal any non-polar components. GC-MS analysis of the least-polar TLC fraction ($R_f \sim 0.7$; benzene:methanol 3:1; silica) resulted in a complex, unresolved gas chromatogram and extremely complex mass spectra; no identification of specific compounds was feasible. Mass spectra of all TLC fractions (Figure 3-7) further indicate the complex nature of the sea-

water fulvic acid.

The fact that ions were observed at 30°C and that the maxima in the distillation curves was below 200°C indicates that non-pyrolytic components of the methylated fulvic acid are sufficiently volatile to be observed by mass spectrometry.

The lack of observable products in the reflux extract at the sensitivity used, and the results of methylation-extraction-TLC-GC-MS analyses indicate that loosely bound non-polar compounds are absent in seawater fulvic acid or present at levels at least an order of magnitude lower than in soil fulvic acids. This may reflect the form in which these materials exist in the sea. In contrast to soils, organic matter in the sea occurs in solution at micromolar concentration and far from being saturated with even non-polar organic compounds. Therefore, the incorporation of non-polar molecules into the hydrophobic sites of micelles is far less likely than in soils, where the concentration of organic matter is higher and organic compounds is further concentrated on surfaces.

Hydrolysis of the fulvic acid sample allowed the identification of some structural components which are bound through amide and ester linkages. The amino acids in Table 3-2 account for 0.6% of the total fulvic acid by weight and 1.1% of the FA nitrogen. Glycine, serine, alanine, aspartic acid and glutamic acid predominate, lesser amounts of proline, threonine, valine and leucine are also present. Only acidic and neutral amino acids are observed in the seawater fulvic acid hydrolyzate, neither basic amino acids, β-alanine, γ-aminobutyric acid, nor ornithine were observed. Aspartic and glutamic acid,

glycine and alanine predominate in fulvic acid from soil (Khan and Sowden, 1971; Khan and Sowden, 1972) and lake sediments fulvic acids (Kemp, 1974; Ishiwatari, 1971), and in humic substances of marine sediments (Degens and Mopper, 1975). These amino acids are abundant in the exudates of some algae (Helebust, 1974); this may partially explain their predominance in seawater fulvic acid.

The incorporation of amino acids into browning reaction products proceeds through Amadori rearrangement of Schiff bases formed by reactions of amino acid amine groups with sugar carbonyls (Hodge, 1953). Amino acids cannot be recovered from these products by hydrolysis. If humic substances in seawater are the result of browning reactions between sugars and amino acids (Kalle, 1966; Nissenbaum, 1974; Jackson, 1975), the low recovery of amino acids obtained in this study may be the result of the incorporation of the amino acids into structures from which they cannot be regenerated. Basic amino acids make up a significant fraction of sedimentary humic substance amino acids (Kemp, 1974; Ishiwatari, 1971; Degens and Mopper, 1975) and soil fulvic amino acids (Khan and Sowden, 1971; Khan and Sowden, 1972). Since they have more amine groups, they are more likely to undergo this irreversible reaction and, therefore, would be recovered in much lower yield. On the other hand, the acidic amino acids have more carboxylic acid groups and are more likely to be incorporated through amide or ester linkages which, in turn, would be subject to cleavage by hydrolysis.

Differences in amino acid composition observed between the seawater fulvic acid and fulvic acids from soils and sediments may in part reflect the different

isolation procedures used. The method employed in this study for the adsorption of organic matter from seawater (Chapter 1) would not recover amino acids because of their highly hydrophilic character (Rohm and Haas, 1969). However, base extraction which is generally used to isolate humic substances from soils and sediments would recover free amino acids and proteins along with the humic substances.

Methylene chloride-soluble basic materials, accounting for 2% of the fulvic acid weight, were recovered upon acid hydrolysis of the fulvic acid sample. These materials were so volatile that they evaporated from the mass spectrometer probe at room temperature before mass spectra could be obtained. GC-MS analysis of this material was then performed after trifluoroacetic acid anhydride treatment, which produces volatile trifluoroacetyl amides from primary and secondary amines, and gave evidence of several volatile components which were not identified. Further characterization was thwarted by decomposition of the sample.

The source of these basic materials is uncertain. Decarboxylation of amino acids to amines is an unlikely source since the decarboxylation products of aspartic acid and glutamic acid, γ-amino butyric acid and β-alanine, were not observed in the amino acid analysis. Amines are present in marine organisms as osmoregulatory agents (Craigie, 1974). Incorporation of such amines into seawater fulvic acid through amide bonding may account for the recovery of amines by acid hydrolysis.

Most of the nitrogen functions in the seawater fulvic acid remain unidentified. They appear to be present either in linkages which are not cleaved by acid hydrolysis, such as heterocyclic structures and polyfunctional amines bonded through other linkages, or as compounds such as amino sugars which would not have been observed by the analyses performed. Since they are important constituents of zooplankton exoskeletons, the presence of amino sugars in seawater fulvic acid is not unlikely.

Methylene chloride-soluble acidic and phenolic hydrolysis products, accounting for 16% of the seawater fulvic acid weight, display broad unresolved gas chromatograms (Table 3-1). This suggests either the complex composition of the samples or the decomposition of these materials during GC analysis. Mass spectra (Figure 3-10) indicate sufficient complexity to account for the broad unresolved gas chromatograms. Since no improvement in the gas chromatograms was observed when special precautions were taken to retard decomposition (on-column injection on glass column; silylation of the sample) the complexity of the samples appears to be responsible for the observations. Perhaps further separation and improved GC resolution would allow identification of some fulvic acid components. It should be noted that most of the material was eluted from the SE-30 column after the column temperature reached 200°C. SE-30 is a non-polar liquid phase and separates mainly according to volatility. Since methyl stearate and heneicosane are eluted at about 200°C under the conditions used, most of the components in the methylated acid and phenol samples are less volatile than these two compounds.

The discussion so far has indicated that the complexity of composition is a major obstacle in the structural elucidation of marine fulvic acid. Because of a similar complexity, methods for the study of soil humic substances either separate it into its constituents (Barton and Schnitzer, 1963; Ogner and Schnitzer, 1971; Khan and Schnitzer, 1971) or simplify the mixture chemically for easier analysis. Because of the very limited sample size available for this investigation (350 mg), the latter approach was adopted.

The most successful techniques for simplifying soil humic substances employ basic permanganate or other strong oxidants which produce aromatic acids from highly aromatic, cross-linked structures (Review: Schnitzer and Khan, 1972). Weak oxidative procedures may increase the complexity by partial and incomplete oxidation while strong oxidizing agents may lead to destruction of aliphatic components, especially branched or olefinic ones. That seawater fulvic acid is highly aliphatic and of low aromaticity is shown by the data of Chapters 1 and 2. Indeed, attempts to apply strong oxidative procedures to seawater humic substances result in complete destruction of the sample (Richard A. Kerr, URI, personal communication).

A reductive rather than an oxidative approach to simplify the seawater fulvic acid was considered more promising. The seawater fulvic acid is from an oxidizing environment and much of the complexity observed may arise from partial oxidation (chemical or biochemical). The effect of this natural oxidation may be partially reversed through chemical reduction. It would be desirable to convert the polyfunctional structures of fulvic acid to hydro-

carbons which are amenable to analysis and provide information on the carbon skeletal arrangement.

Reductive procedures used in studies of soil humic substances include Zn-dust distillation which is carried out at high temperatures (400-550^{o}C). It may lead to pyrolysis and carbon structural rearrangement (Schnitzer and Khan, 1972). Na amalgam reduction has been useful for the identification of phenolic structures (Stevenson and Mendez, 1967), but low yields make this procedure unappealing.

Hoering (1971) succeeded in converting polar fractions of shale organic matter to hydrocarbons. Lithium aluminum hydride (LAH) converts carbonyl groups to alcohols. After transformation into iodides these are further reduced to hydrocarbons with LAH. This promising method was attempted in this laboratory to convert humic substances to hydrocarbons; however, high hydrocarbon blanks from the LAH could not be reduced to acceptable levels and the method was, therefore, abandoned.

Many investigators have used high pressure hydrogenation and hydrogenolysis to reduce humic substances (Gottlieb and Hendricks, 1946; Kukharenko and Savelev, 1951; Kukharenko and Savelev, 1952; Murphy and Moore, 1960; Felbeck, 1965). Many of these investigations resulted in the formation of colorless oils but in only one case were products identified; a C_{25} or C_{26} n-alkane was identified in the reduction products of humic acids from a muck soil (Felbeck, 1965).

High pressure catalytic hydrogenation reduces carbonyl groups to alcohols. Further reduction to hydrocarbons occurs only in special cases, e.g. benzylic

alcohols (Augustine, 1965). Two catalysts are widely used: copper chromite which does not reduce aromatic rings and Raney nickel which reduces aromatic rings to cyclohexanes under the conditions required to reduce carboxylic acids. The reduction of aromatic rings can lead to mixtures of stereochemical isomers which complicates the interpretation of results. Other catalysts may be used to promote hydrogenolysis of functional groups to form hydrocarbons but they have not been successfully applied to soil humic substances (Felbeck, 1965).

High pressure hydrogenation of methyl esters to alcohols using copper chromite requires lower temperatures and pressures than the reduction of carboxylic acids. Furthermore, the addition of barium to the catalysts increases its activity (Augustine, 1965).

The methylation of the fulvic acid followed by high pressure hydrogenation with Cu-Ba chromite catalyst was chosen to provide the most complete reduction possible under the mildest conditions. Esters, acids, aldehydes, ketones and double bonds are reduced under the conditions chosen but aromatic rings, amides and amines are not reduced (Augustin, 1965). The expected reduction products were alcohols.

The further reduction of these alcohols to hydrocarbons is more easily accomplished after the formation of derivatives (e.g. tosylates of mesylates) or displacement of the hydroxyl with halides. Dibromotriphenylphosphorane was chosen to convert the alcohols to bromides since it is a powerful but highly specific reagent (Wiley et al., 1964; Schaefer and Weinberg, 1965a; Schaefer and Weinberg, 1965b; Horner et al., 1959). It is also capable of displacing

phenolic hydroxyls to form aryl bromides (Horner et al., 1959), and of cleaving ethers to form alkyl bromides (Anderson and Freenor, 1964).

Further reduction was then carried out in basic medium with a palladium catalyst. These conditions are sufficient to reduce alkyl and aryl bromides, as well as any olefins produced by dehydrohalogenation, to hydrocarbons (Augustine, 1965).

Thus, the overall hydrogenation-bromination-hydrogenation scheme (Figure 3-2) is, in principle, capable of converting esters, ketones, aldehydes, alcohols, phenols, olefins, and ethers to saturated or aromatic hydrocarbons; amides and amines would not be reduced. In actual fact, however, constituents containing several functional groups will give a low yield because of the accumulated effect of incomplete yields in the reduction of each functional group. Also, steric hinderance may inhibit the reduction of some functional groups. Indeed, as the ir spectrum of the reduction products (Figure 3-11) shows, some ester functions are still present.

The GC conditions in this study permit the identification of hydrocarbons with 8 to 32 carbons. In spite of this limitation, interesting structural information was obtained by applying the hydrogenation-bromination-hydrogenation scheme to seawater fulvic acids.

The alkanes in the reduction products (Figures 3-14 and 3-19) have a high even to odd carbon chain predominance,

$$\frac{C_{16} + C_{18}}{2C_{17}} \simeq 5.0 \text{ ,}$$

a small amount of branched structures, and a C_{12} to C_{22} range with the maximum in the distribution at C_{18} or C_{20}. These are all characteristics of the fatty acid distribution of marine organisms (Williams, 1965; Schultz and Quinn, 1972; Parker, 1969; Blumer et al., 1969) and suggests that these are the source of the alkanes in the reduction products. This is further supported by the observation of a series of fatty acid methyl esters in alumina chromatography fraction 5 of the reduction-bromination-reduction products (Figure 3-19). These esters may not have been reduced because they were shielded by the surrounding fulvic acid structure or were too hindered to be adsorbed on the hydrogenation catalyst. Their presence as methyl esters in the reduction products suggests that the high pressure hydrogenation and bromination steps rearranged the structure sufficiently to allow cleavage of ester linkages in the final reduction step through transesterification in the anhydrous basic methanol.

The distribution of fatty acid moieties in the seawater fulvic acid, suggested by the alkane distribution after reduction, is quite different than that in soil fulvic acids. These have a carbon range from C_{16} to C_{35} with the maximum in the distribution at C_{24} (Figure 3-20) (Schnitzer and Ogner, 1970). The fatty acids in soil humic substances represent contributions from living organisms and from plant and insect waxes (C_{26} to C_{38} even acids). That C_{26} to C_{38} hydrocarbons are not abundant in the seawater fulvic acid reduction products indicates that land-derived organic matter is not contributing significantly to the marine humic substances.

The origin of the aromatic hydrocarbons (Figures 3-14, 3-15, 3-16, 3-17) in the reduction products of seawater fulvic acid is less clear. They may represent preformed compounds, such as phenols from brown algae (Sieburth, 1965; Craigie and McLachlan, 1964; Sieburth and Jensen, 1968; Sieburth and Jensen, 1969), which had been incorporated into the fulvic acid and were recovered as hydrocarbons after reduction.

Cross-linking and cyclization of olefins can lead to aromatic structures which usually contain disubstituted aromatic rings (Edmunds and Johnstone, 1965; Andresen, 1972). However, this source for the aromatic structures in the reduction products is unlikely since only monosubstituted benzenes are observed and since the C_2 to C_4 α-substituents are difficult to explain on this basis. The cyclization of terpenoid structures would not give rise to the ethyl, propyl and butyl branching observed. The lack of even:odd carbon preference indicates that the cyclization of unsaturated fatty acids is not involved.

The possibility exists that these aromatic compounds arise from cyclization and aromatization during the reduction of the fulvic acid. However, differences observed in the complexity of the aromatics present in the Sargasso and coastal samples (Figures 3-13c and 3-18c) suggest that the aromatic structures are originally present in the seawater fulvic acid.

The contribution of these aromatic compounds from the Amberlite XAD-2 resin must be considered. However, the fulvic acid blank showed no ir absorption between 2800 cm^{-1} and 3100 cm^{-1} (C-H stretching region) and demonstrates that insignificant amounts of organic matter are present. Furthermore, structures such

as those present in the reduction products are not observed by GC-MS analysis of concentrated Amberlite XAD-2 contaminants (personal communication, Dr. R. A. Hites, Massachusetts Institute of Technology).

The highly aliphatic character of seawater fulvic acid is suggested by the data of Chapters 1 and 2. The products of reduction of seawater fulvic acid indicate that these aliphatic moieties may represent marine lipids which are incorporated into the structure. The alkanes and aromatic hydrocarbons in the reduction products account for 3% of the carbon in the fulvic acid; however, considering the multistep procedure and also the sterically hindered and polyfunctional nature of the fulvic acid, such a low yield is not unexpected. The lack of identifiable lipids in the products of reflux extraction, methylation-TLC, or acid hydrolysis suggests that the lipids are bonded through hindered ester or amide functions or through ether linkages.

Summary

(1) Seawater fulvic acid is an extremely complex mixture of polyfunctional organic compounds containing both polar and non-polar moieties.

(2) Nitrogen is present mainly in forms other than hydrolyzable amino acids, such as polyfunctional amines (e.g. amino sugars) or heterocycles. Some volatile bases are recovered upon hydrolysis.

(3) Non-polar moieties are incorporated mainly through covalent chemical bonds rather than through weaker interactions, such as in hydrophobic sites of micelles.

(4) Simple fractionation methods, such as TLC and GC, are not sufficient for resolving individual components of the fulvic acid; however, the fact that volatile compounds are produced by derivatization indicates that extensive

fractionation schemes combined with GC-MS may be a fruitful approach to structural elucidation.

(5) A reduction procedure was developed to convert polyfunctional molecules to hydrocarbons. This method may be generally applicable to the structural elucidation of other complex organic mixtures.

(6) Hydrocarbons produced by reduction of seawater fulvic acid indicate that marine lipids are important structural components and that terrestrial sources are minor, even in coastal samples.

(7) An unusual series of aromatic structures was observed in the reduction products; the source of these is unknown.

Figure 3-1. Fractionation scheme for CH_2Cl_2-soluble acid hydrolysis products.

Figure 3-2. Scheme for reduction of methylated fulvic acid to hydrocarbons.

EXPERIMENTAL CONDITIONS

FULVIC ACID METHYL ESTERS

↓ 5,000 psi; Copper-Barium-Chromium Oxide catalyst; 180°C; 36 hours; Methanol

TOTAL PRODUCT

↓ Dibromotriphenylphosphorane; N_2; 105°C; 24 hours; Acetonitrile

ETHER SOLUBLE PRODUCT

↓ 1 Atm. H_2; 10% $Pd/BaCO_3$; 12 hours; 1% KOH/Methanol

CYCLOHEXANE SOLUBLE PRODUCT

↓ Alumina Chromatography; 0, 2, 5, 10, 20, 100% CH_2Cl_2/ Pentane

5 FRACTIONS ANALYZED BY GC AND GC-MS

Figure 3-3. Ion compositions from high resolution mass spectrometric analysis of Sargasso Sea fulvic acid superimposed on low resolution mass spectrum (probe temperature 260°C).

5 16 73 2073--125

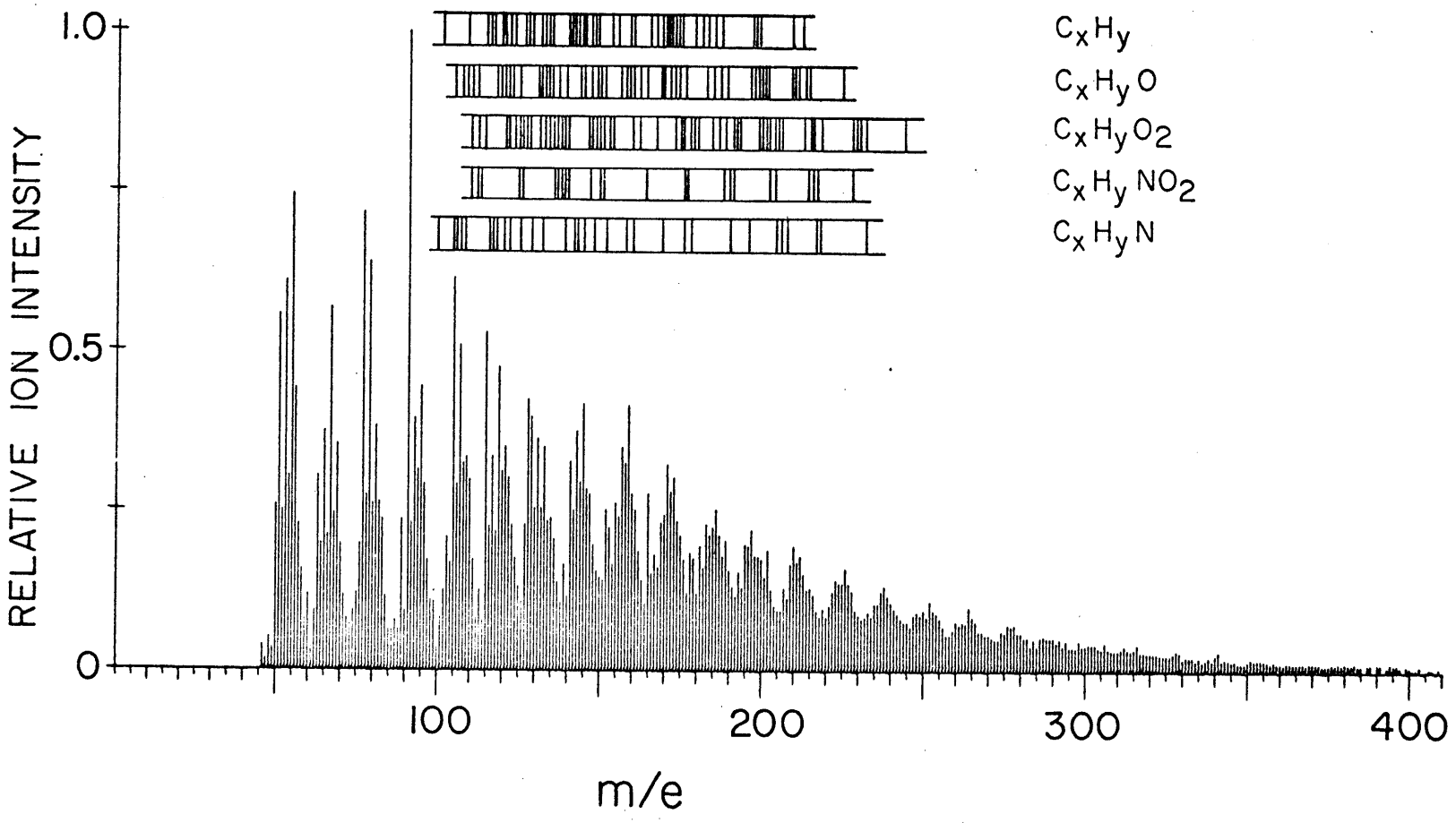
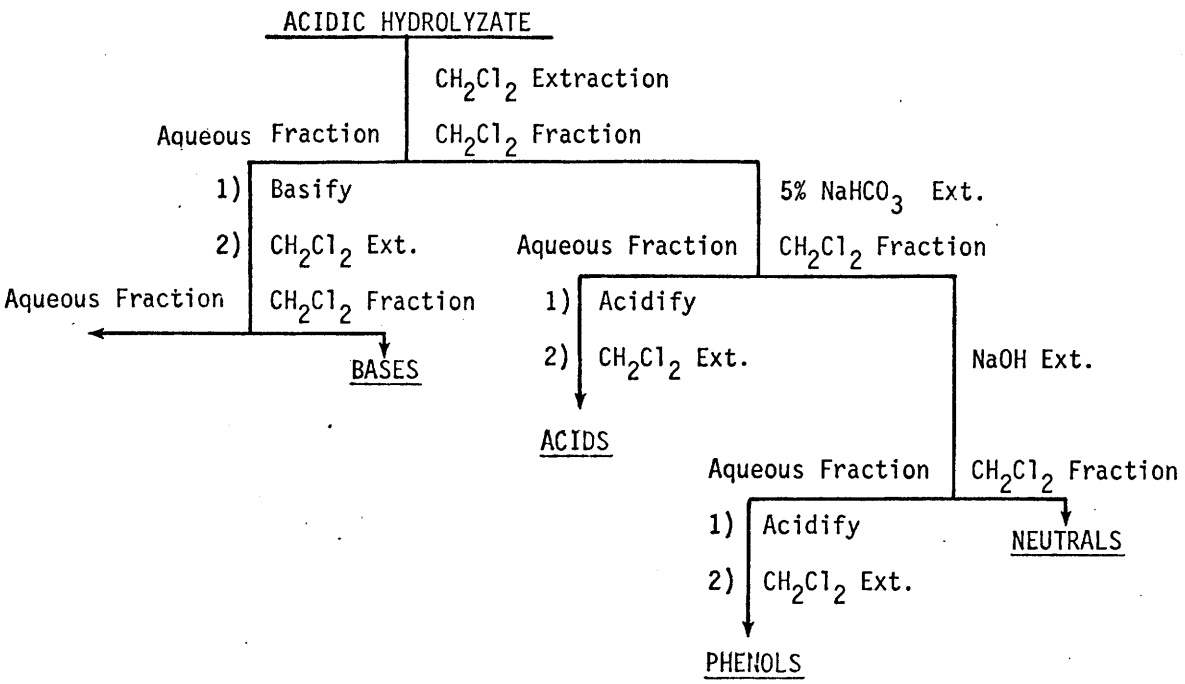


Figure 3-4. Reconstructed gas chromatogram of GC-MS of methylated/silylated Sargasso Sea fulvic acid.

FRACTIONATION SCHEME FOR HYDROLYSIS PRODUCTS



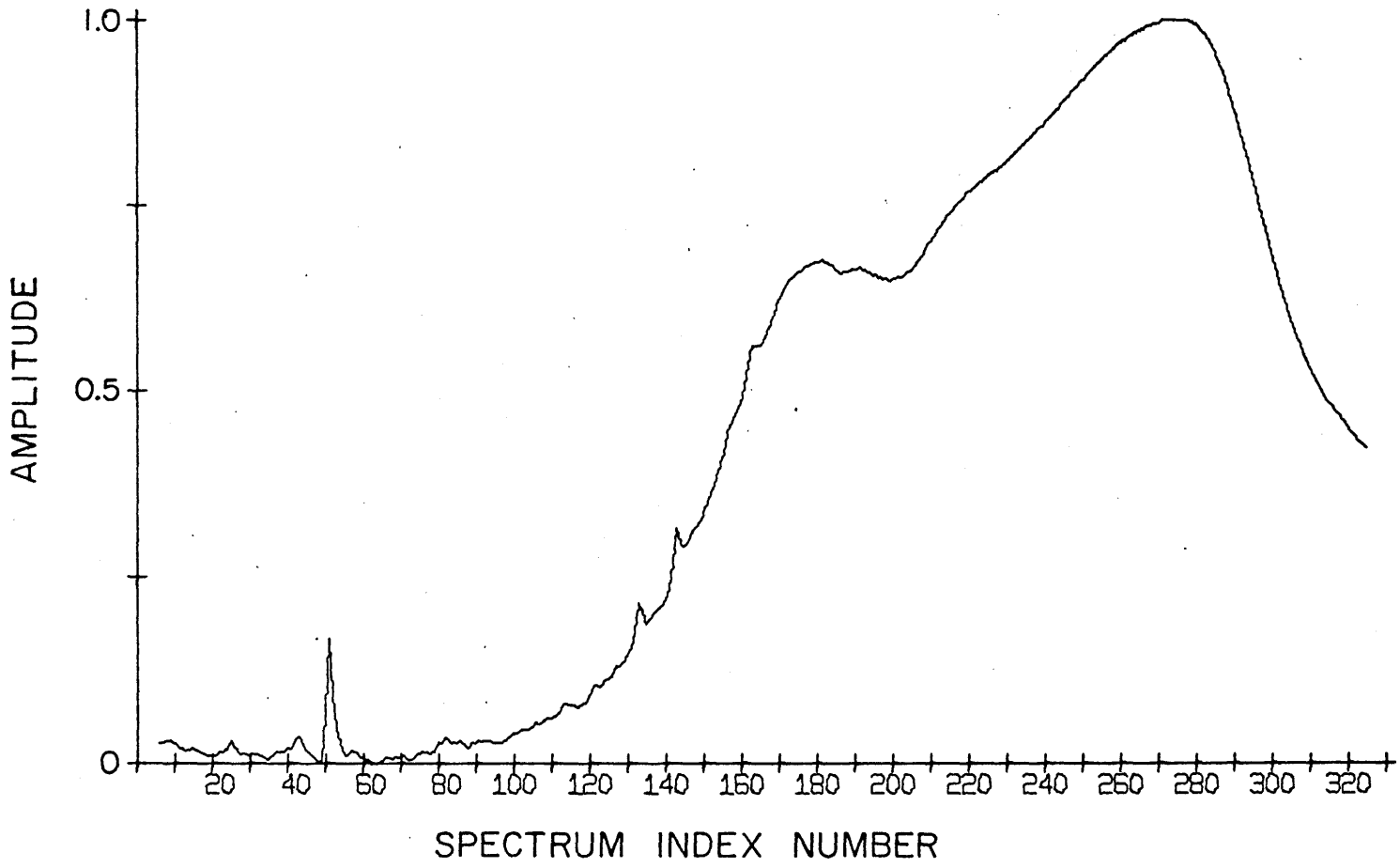

1.0
0.5
0
AMPLITUDE
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
SPECTRUM INDEX NUMBER

Figure 3-5. Mass spectrum from GC-MS of methylated/silylated Sargasso Sea fulvic acid (elution temperature 340°C).

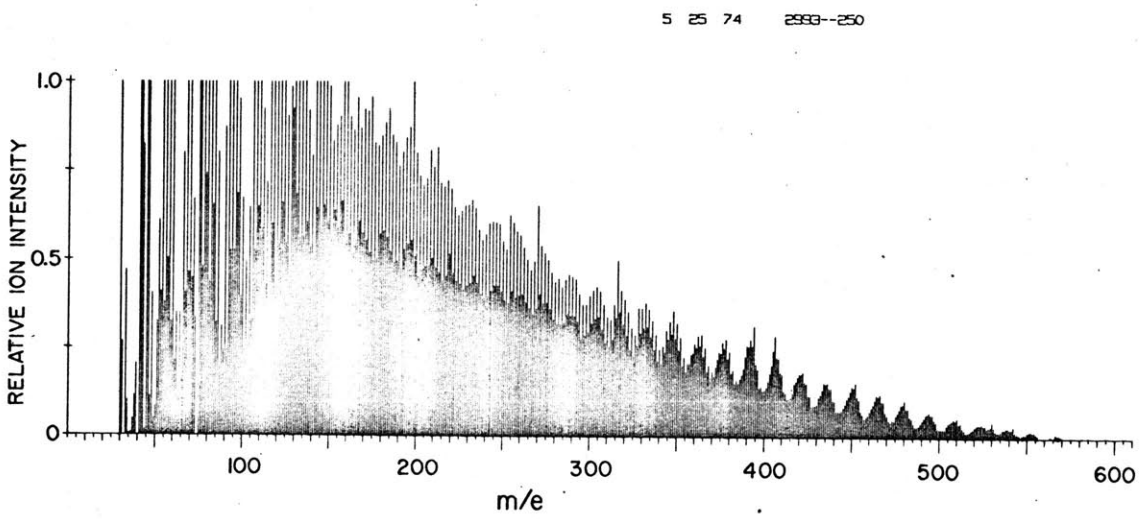

5 25 74 2993--250
1.0
0.5
0
RELATIVE ION INTENSITY
100
200
300
400
500
600
m/e

Figure 3-6. Thin-layer chromatogram of methylated Sargasso Sea fulvic acid (B) and low molecular weight fraction (A). Silica plate was eluted with benzene:methanol (3:1).

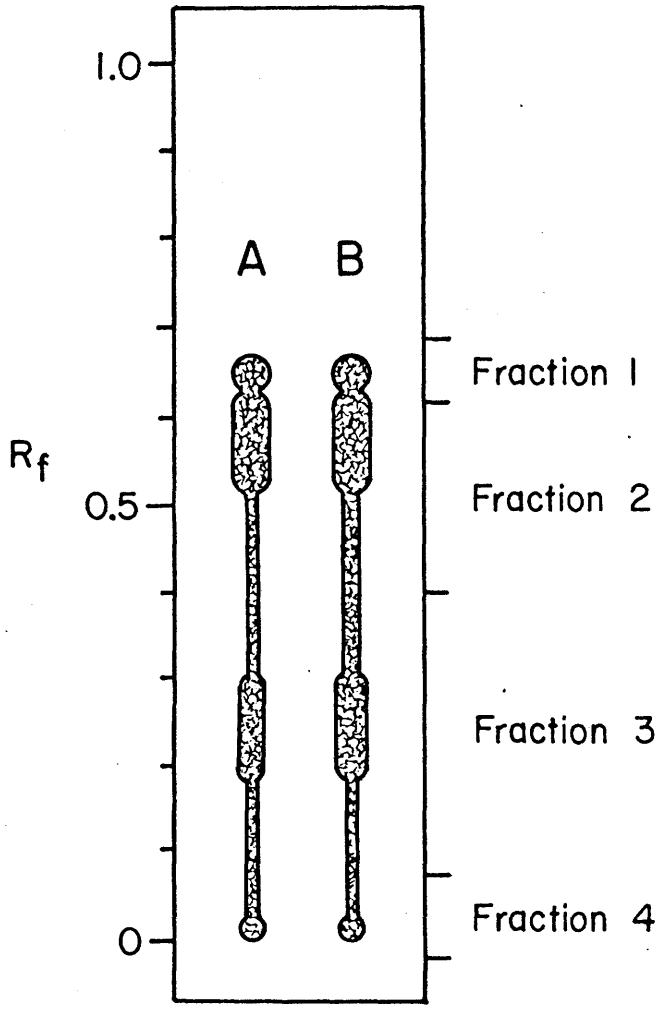

1.0
A
B
Fraction 1
R_f
0.5
Fraction 2
Fraction 3
0
Fraction 4

Figure 3-7. Mass spectra of methylated Sargasso Sea thin-layer chromatogram (see Figure 3-6) fraction 1 (spectrum A, probe temperature 140°C), fraction 2 (spectrum B, probe temperature 190°C), fraction 3 (spectrum C, probe temperature 210°C) and probe background (spectrum D).

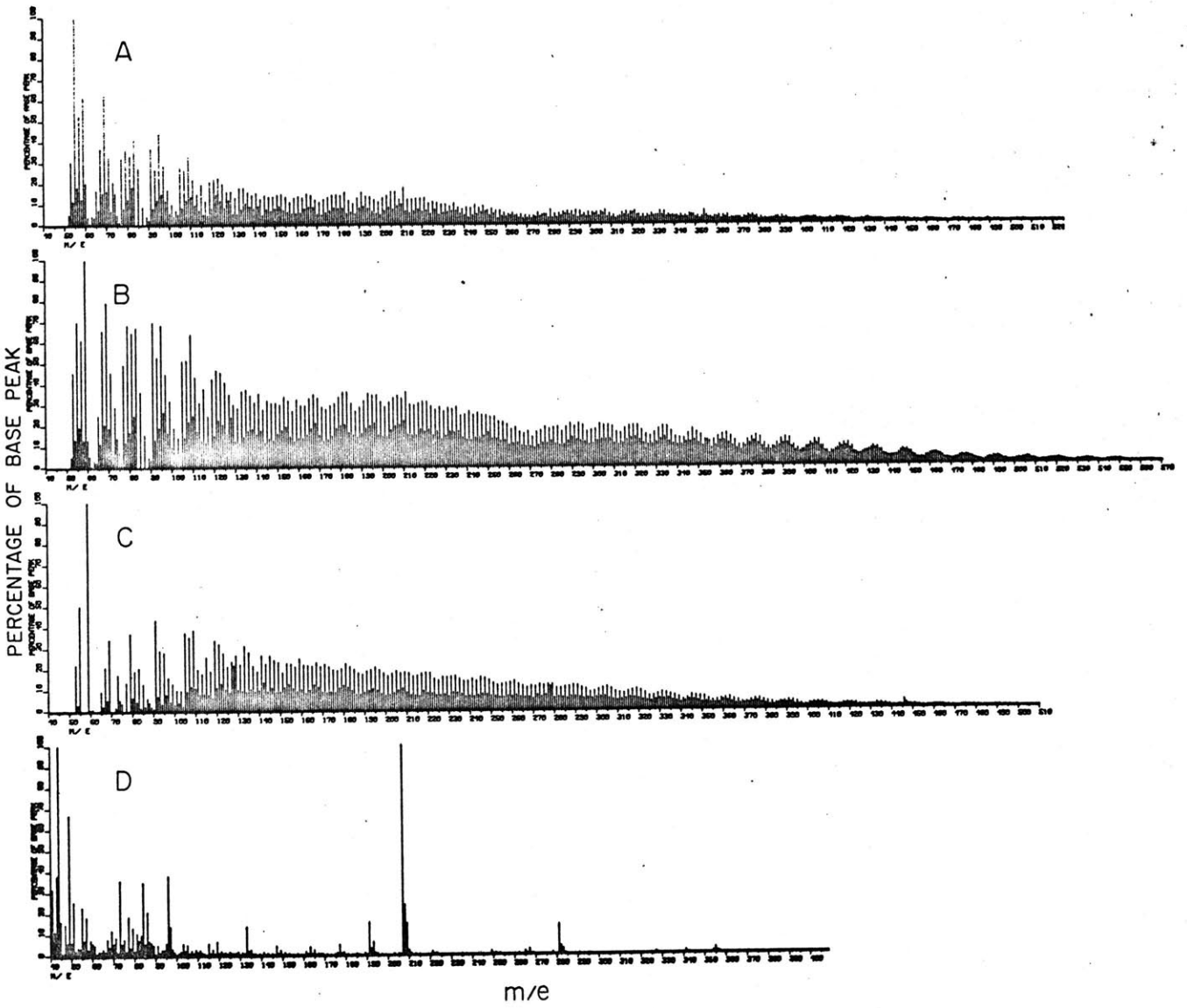

A
B
C
D
PERCENTAGE OF BASE PEAK
m/e

Figure 3-8. Gas chromatograms of derivatized amino acids in hydrolyzate of Sargasso Sea fulvic acid and blank.

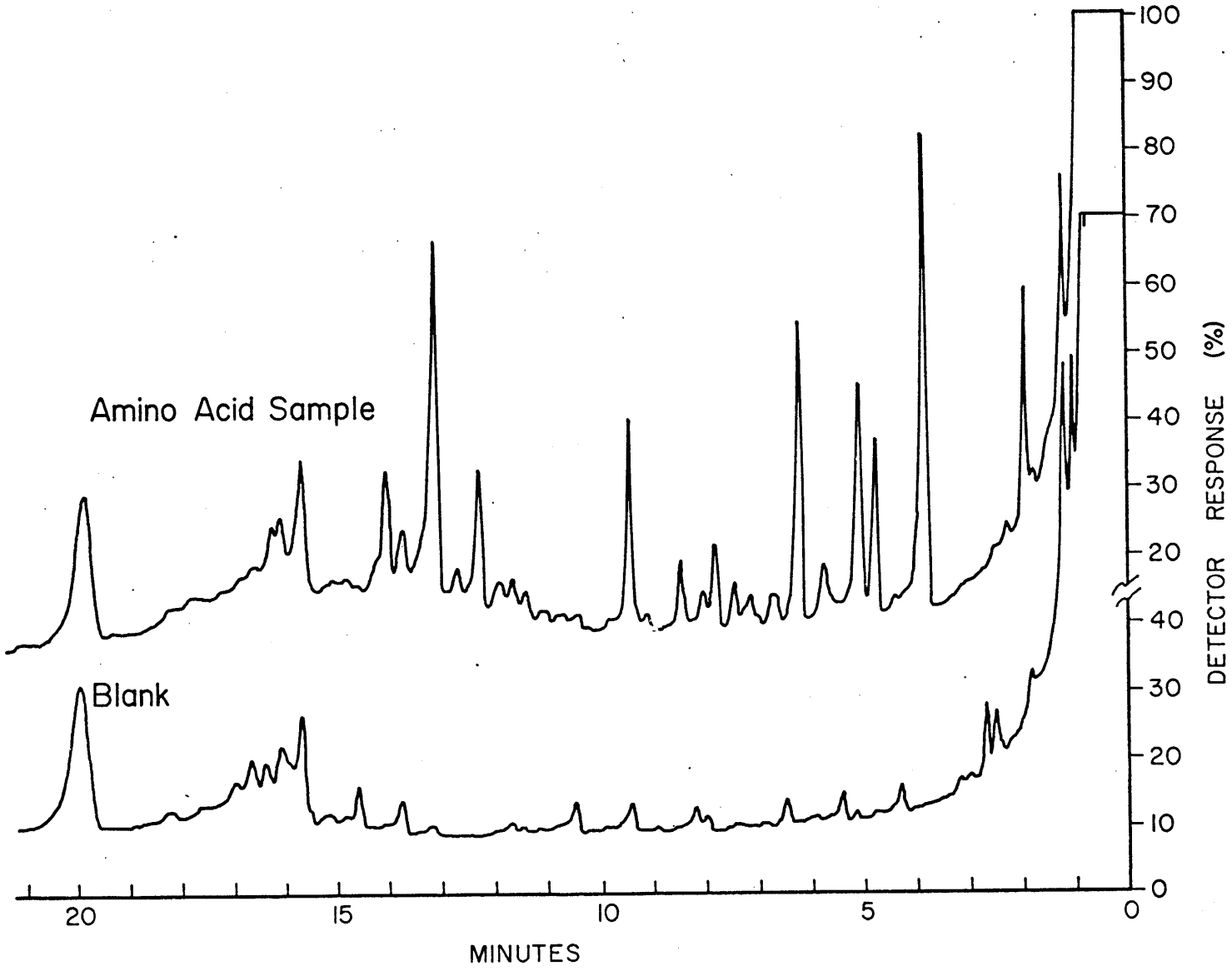

Amino Acid Sample
Blank
100
90
80
70
60
50
40
30
20
40
30
20
10
0
DETECTOR RESPONSE (%)
20
15
10
5
0
MINUTES

Figure 3-9. Reconstructed gas chromatograms (RGC) from GC-MS of standard amino acid solution (upper RGC) and amino acids in hydrolyzate of Sargasso Sea fulvic acid (lower RGC). Peaks marked with (X) indicate contaminants, those marked with (?) indicate unknown compounds.

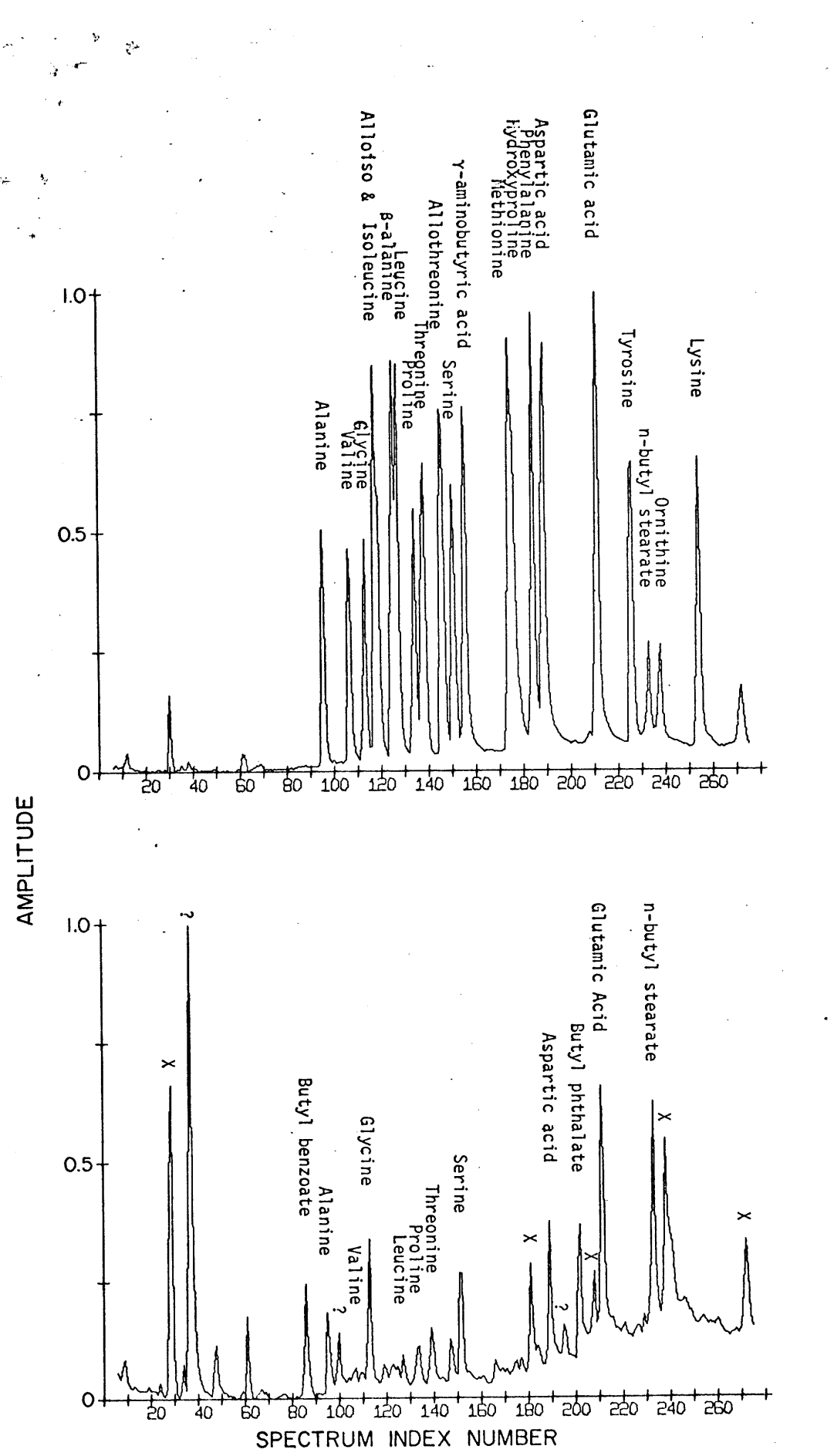

Lysine
Tyrosine
n-butyl stearate
Ornithine
Glutamic acid
Aspartic acid
Phenylalanine
Hydroxyproline
Methionine
γ-aminobutyric acid
Allothreonine
Serine
Threonine
Proline
β-alanine
Leucine
Allo so & Isoleucine
Glycine
Valine
Alanine
1.0
0.5
0
20
40
60
80
100
120
140
160
180
200
220
240
260
AMPLITUDE
n-butyl stearate
Glutamic Acid
Butyl phthalate
Aspartic acid
Serine
Threonine
Proline
Leucine
Glycine
Valine
Alanine
Butyl benzoate
X
?
SPECTRUM INDEX NUMBER

Figure 3-10. Mass spectrum from GC-MS analysis of CH_2Cl_2-soluble acids in hydrolyzate of Sargasso Sea fulvic acid.

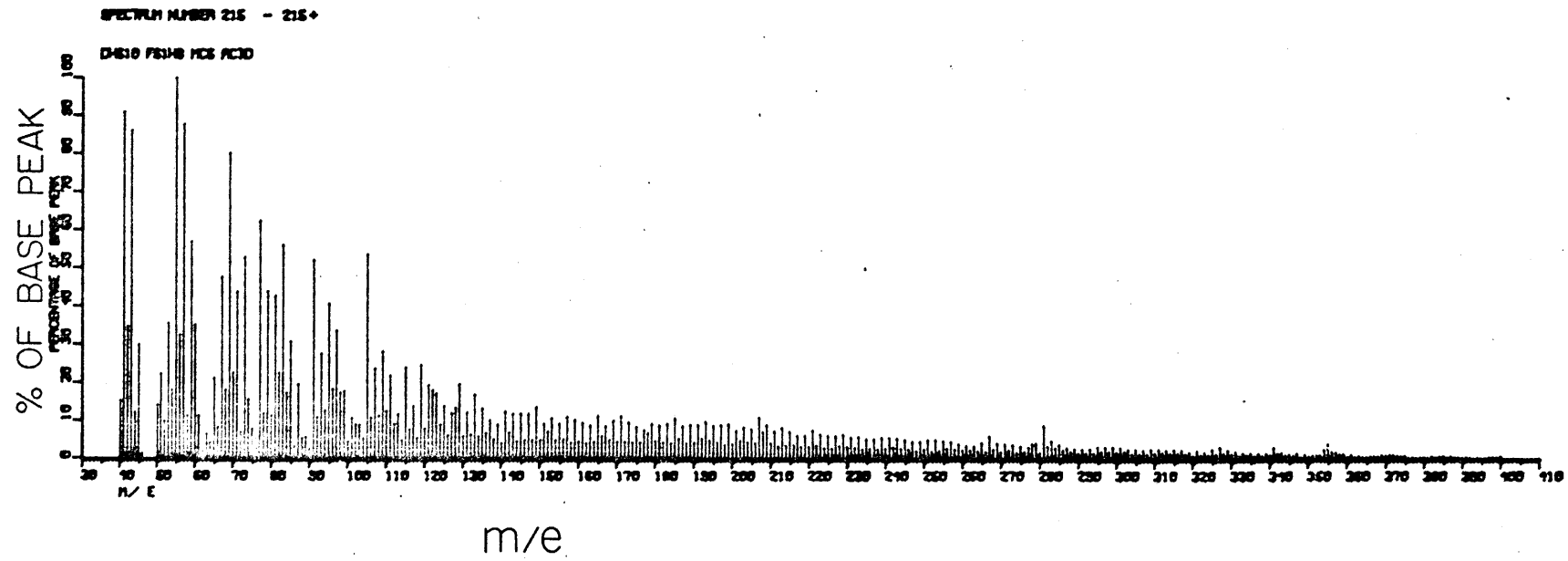

% OF BASE PEAK
m/e

Figure 3-11. Infrared spectra of methylated Sargasso Sea fulvic acid (A) and products of high pressure hydrogenation (B).

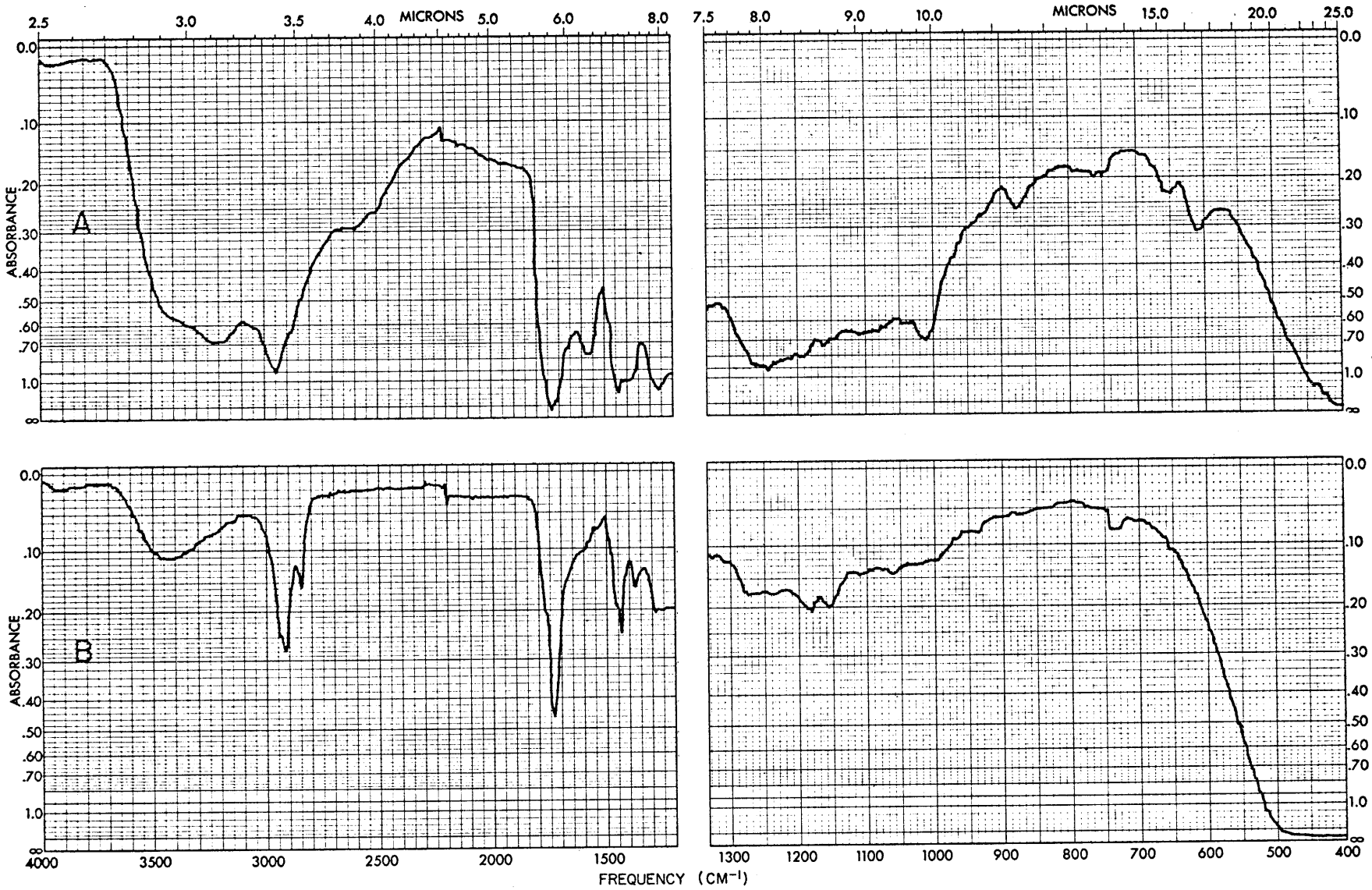

2.5
3.0
3.5
4.0
MICRONS
5.0
6.0
8.0
7.5
8.0
9.0
10.0
MICRONS
15.0
20.0
25.0
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
ABSORBANCE
A
B
4000
3500
3000
2500
2000
1500
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM⁻¹)

Figure 3-12. Gas chromatograms of fraction 1 (see Figure 3-2) from reduction of Sargasso Sea fulvic acid (A), procedural blank concentrated 5x (B) and standard even-numbered n-alkane mixture (C).

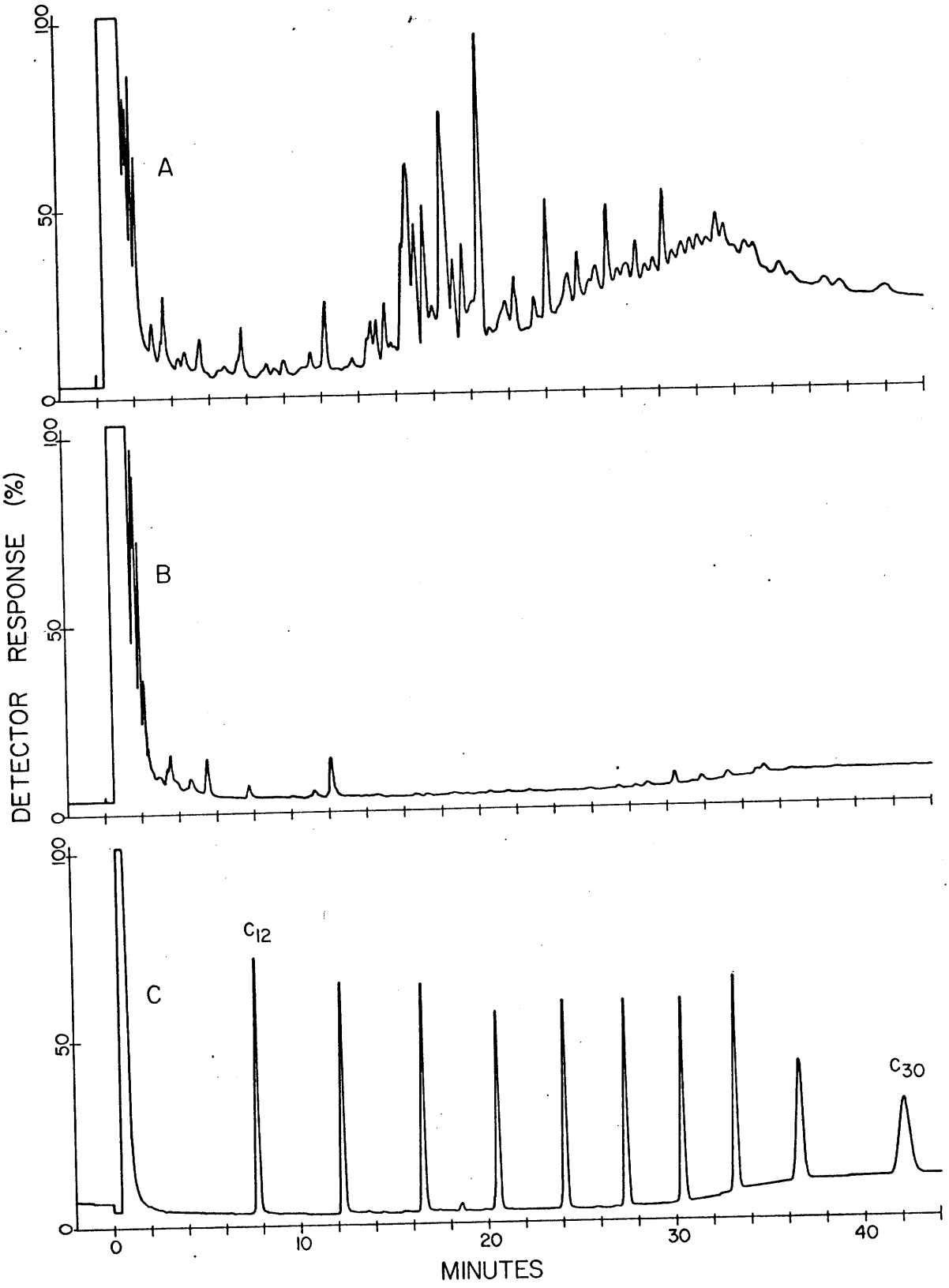

100
50
0
A
B
C
DETECTOR RESPONSE (%)
C12
C30
0
10
20
30
40
MINUTES

Figure 3-13. Reconstructed gas chromatogram (total ion plot), mass plot for alkyl groups (m/e 57, 71, 85, 99, 113), and mass plot for substituted benzenes (m/e 77, 91, 105, 119, 133) of fraction 1 (see Figure 3-2) from reduction of Sargasso Sea fulvic acid.

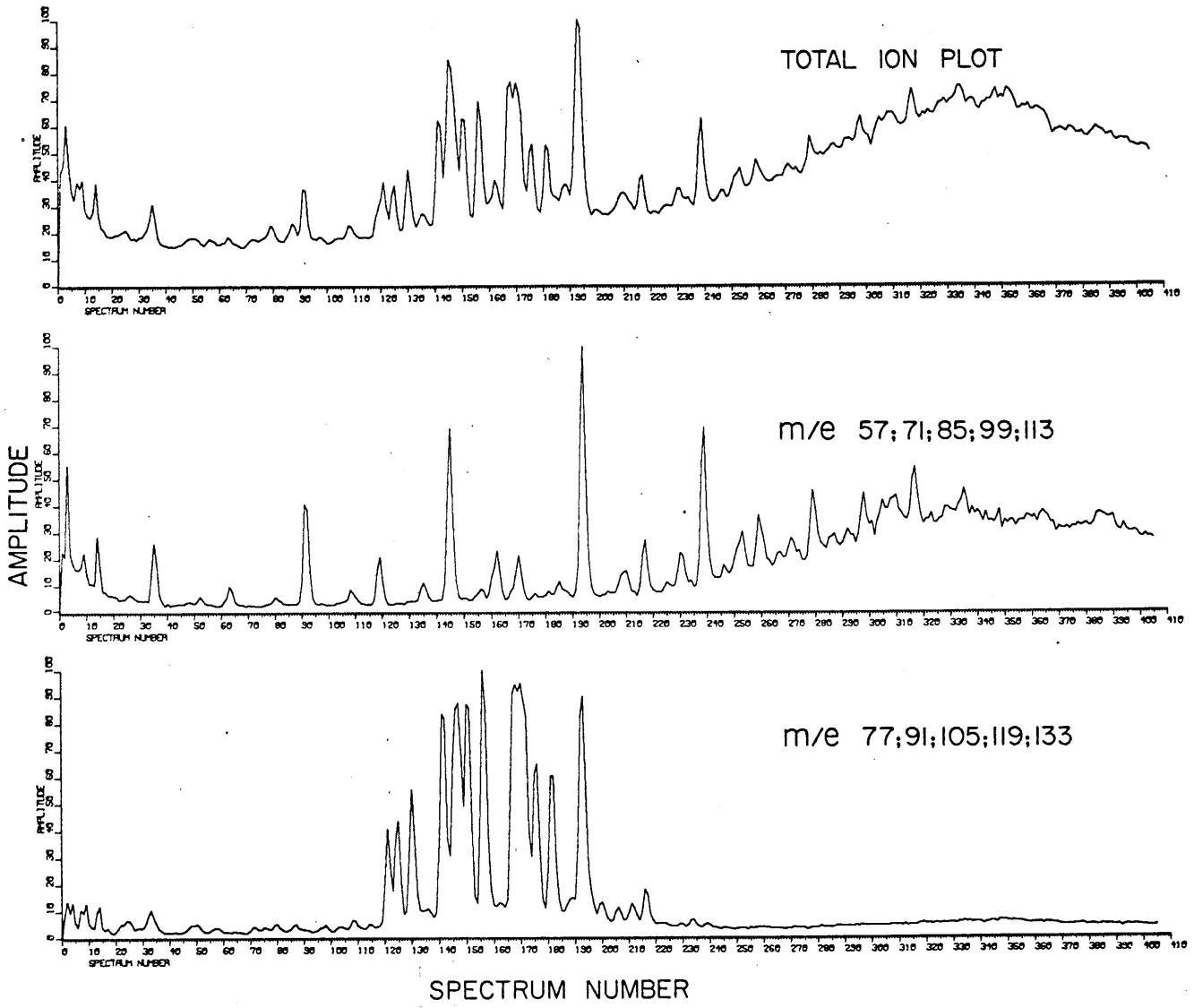
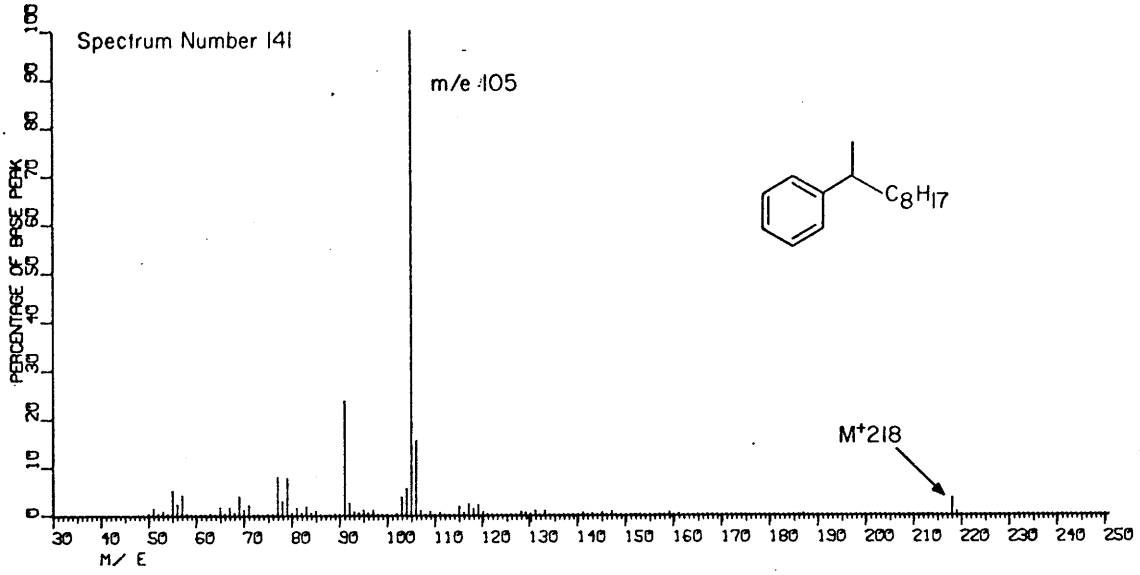
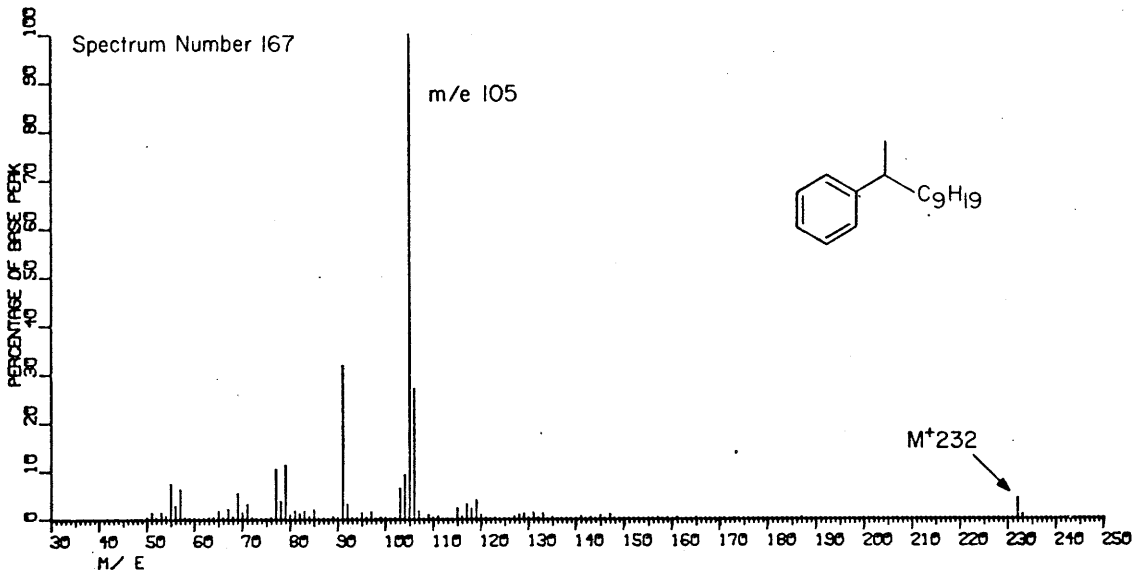

TOTAL ION PLOT
m/e 57;71;85;99;113
m/e 77;91;105;119;133
AMPLITUDE
SPECTRUM NUMBER

Figure 3-14. Mass spectra of α-methylalkyl benzenes in reduction products of Sargasso Sea fulvic acid.


Spectrum Number 141
m/e 105
C_8H_{17}
M^+218
PERCENTAGE OF BASE PEAK
M/ E


Spectrum Number 167
m/e 105
C_9H_{19}
M^+232
PERCENTAGE OF BASE PEAK
M/ E

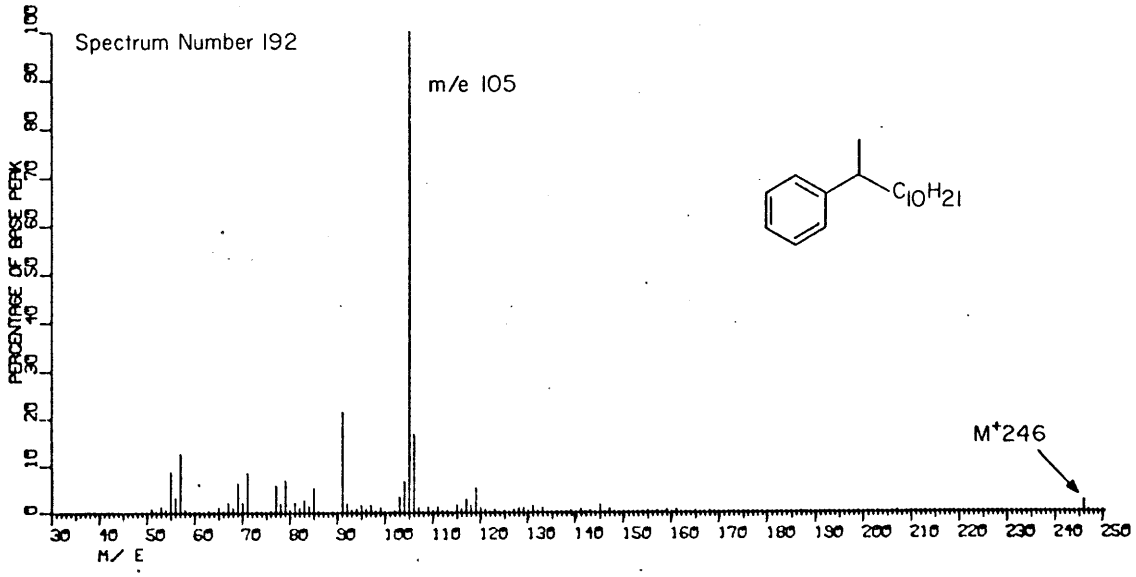
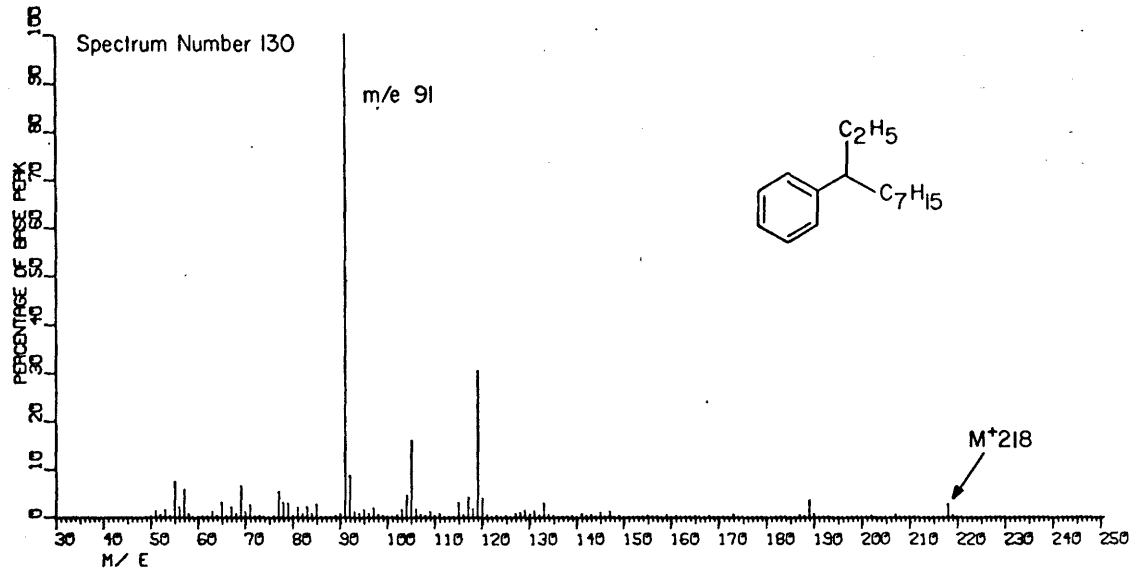
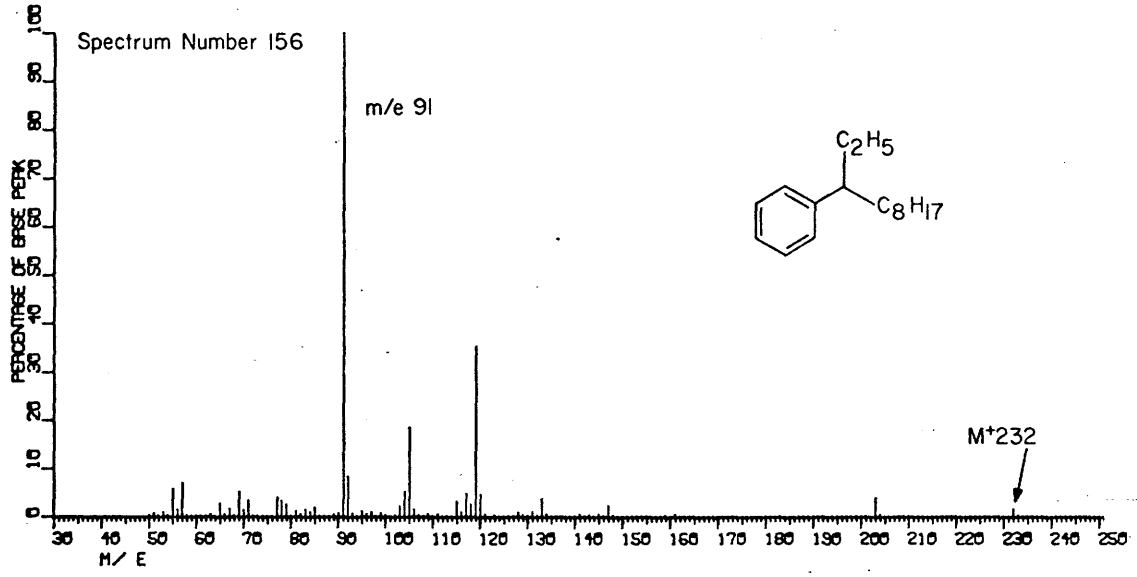

Spectrum Number 192
m/e 105
$C_{10}H_{21}$
M^+246
PERCENTAGE OF BASE PEAK
M/ E

Figure 3-15. Mass spectra of α-ethylalkyl benzenes in reduction products of Sargasso Sea fulvic acid.


Spectrum Number 130
m/e 91
C_2H_5
C_7H_{15}
M+218
PERCENTAGE OF BASE PEAK
M/ E


Spectrum Number 156
m/e 91
C_2H_5
C_8H_{17}
M+232
PERCENTAGE OF BASE PEAK
M/ E

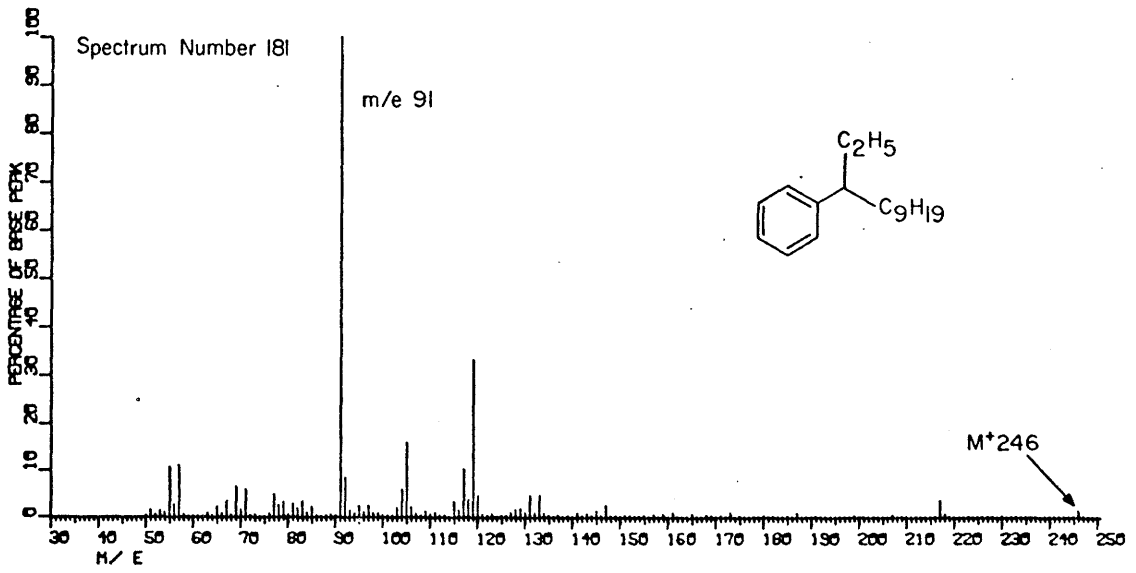
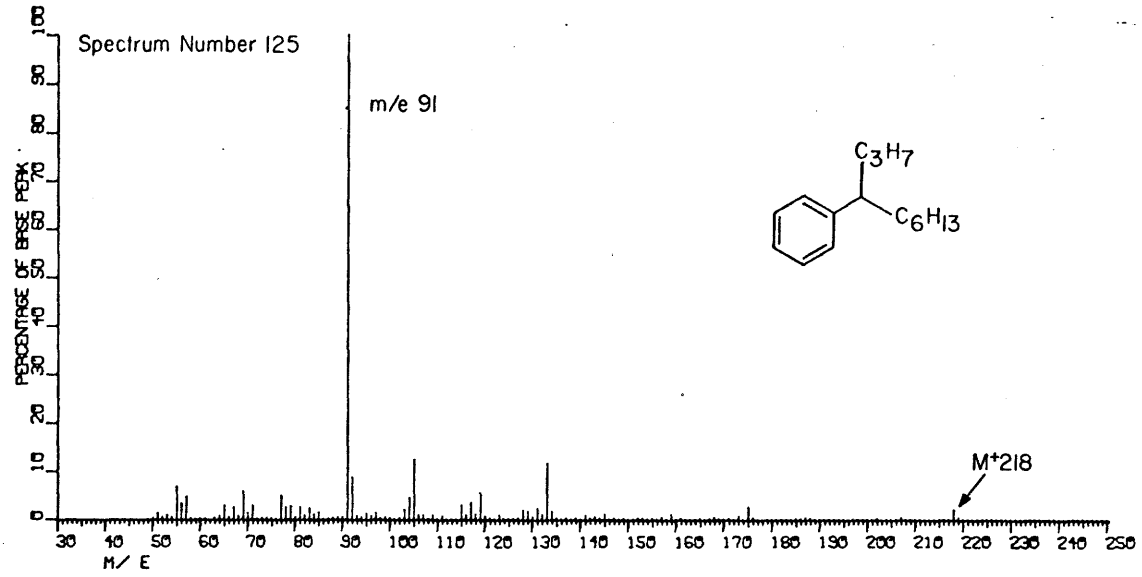
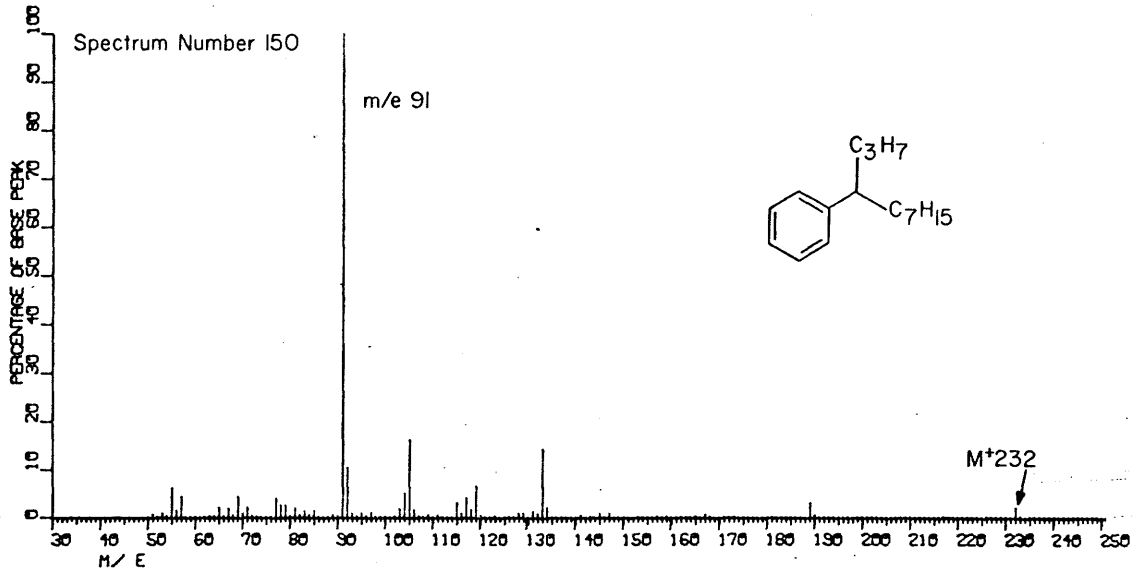

Spectrum Number 181
m/e 91
C_2H_5
C_9H_{19}
M+246
PERCENTAGE OF BASE PEAK
M/ E

Figure 3-16. Mass spectra of α-propylalkyl benzenes in reduction products of Sargasso Sea fulvic acid.


Spectrum Number 125
m/e 91
C_3H_7
C_6H_{13}
M+218
PERCENTAGE OF BASE PEAK
M/ E


Spectrum Number 150
m/e 91
C_3H_7
C_7H_{15}
M+232
PERCENTAGE OF BASE PEAK
M/ E

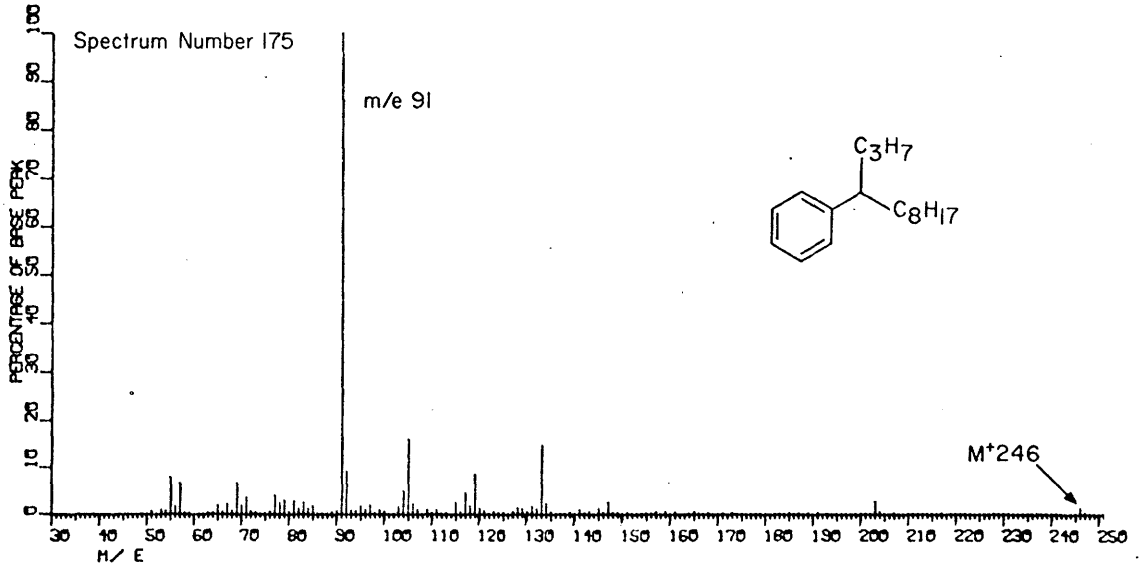
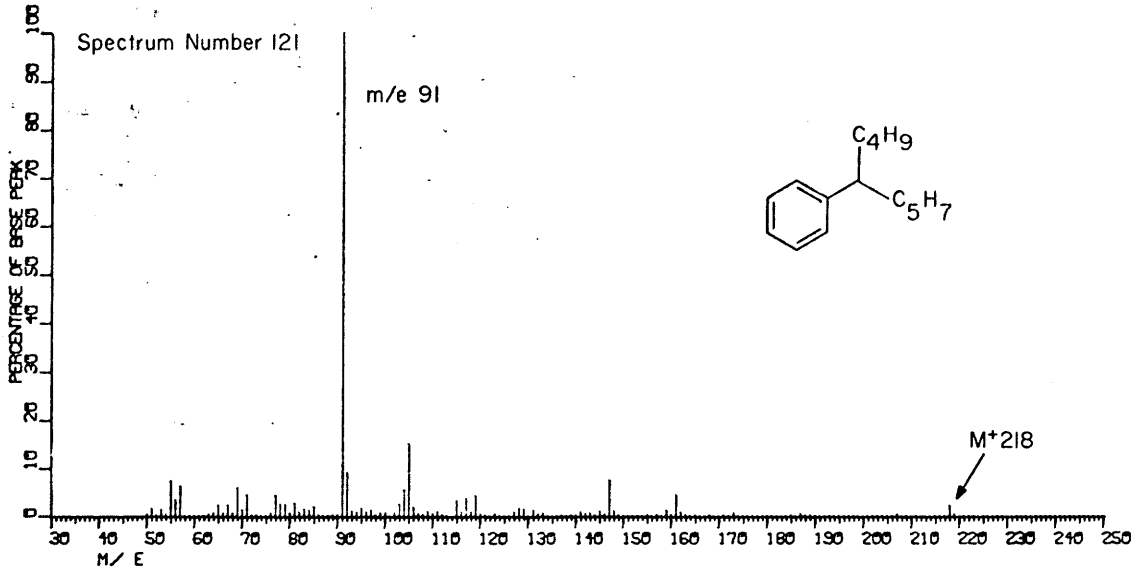
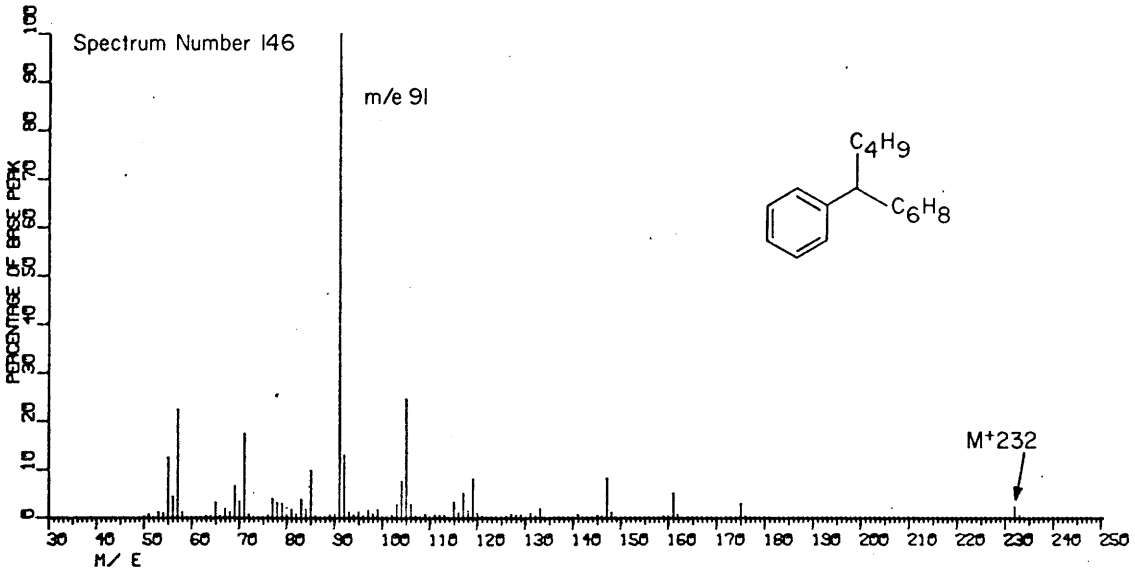

Spectrum Number 175
m/e 91
C_3H_7
C_8H_{17}
M+246
PERCENTAGE OF BASE PEAK
M/ E

Figure 3-17. Mass spectra of α-butylalkyl benzenes in reduction products of Sargasso Sea fulvic acid.


Spectrum Number 121
m/e 91
C_4H_9
C_5H_7
M+218
PERCENTAGE OF BASE PEAK
M/ E


Spectrum Number 146
m/e 91
C_4H_9
C_6H_8
M+232
PERCENTAGE OF BASE PEAK
M/ E

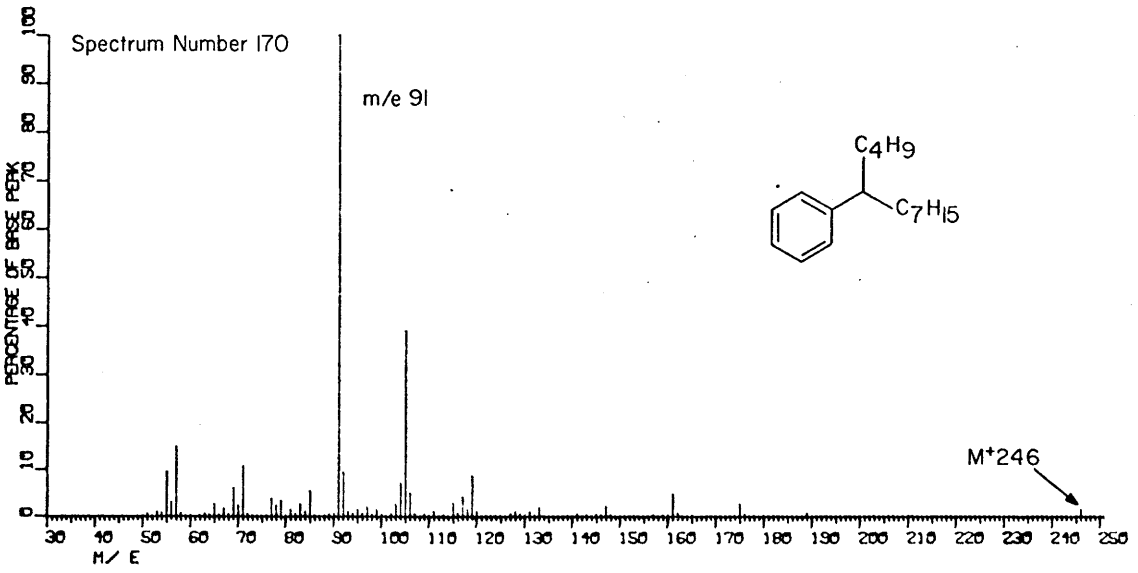

Spectrum Number 170
m/e 91
C_4H_9
C_7H_{15}
M+246
PERCENTAGE OF BASE PEAK
M/ E

Figure 3-18. Reconstructed gas chromatogram (total ion plot), mass plot for alkyl groups (m/e 57, 71, 85, 99, 113), and mass plot for substituted benzenes (m/e 77, 91, 105, 119, 133) of fraction 1 (see Figure 3-2) from reduction of coastal water fulvic acid.

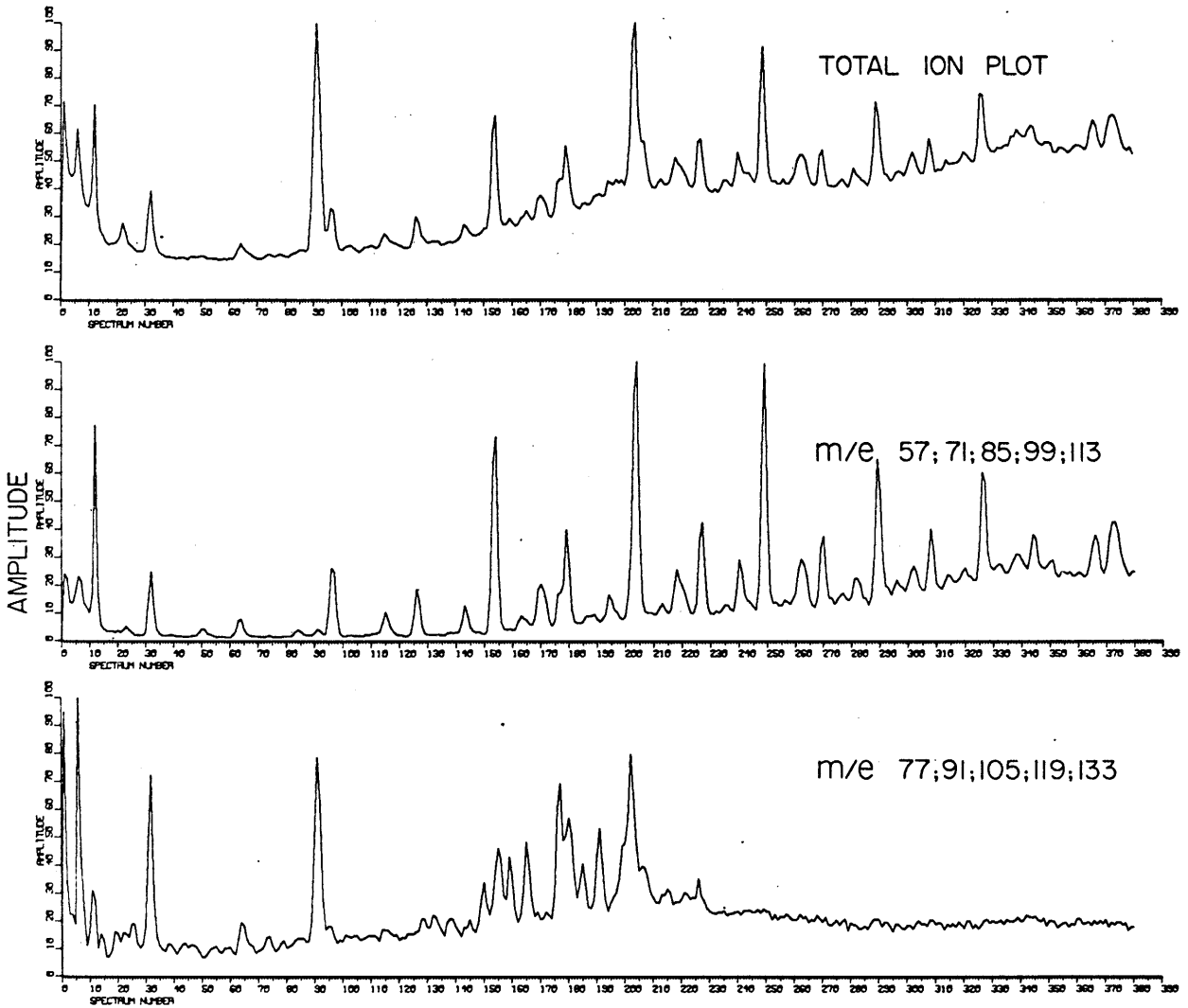

TOTAL ION PLOT
m/e 57;71;85;99;113
m/e 77;91;105;119;133
AMPLITUDE
SPECTRUM NUMBER

Figure 3-19. Gas chromatograms of fraction 5 (see Figure 3-2) from reduction of Sargasso Sea fulvic acid (A), coastal water fulvic acid (B), and procedural blank concentrated 5x (C). Chain length of fatty acid methyl esters are indicated. Peaks marked with (X) indicate contaminants.

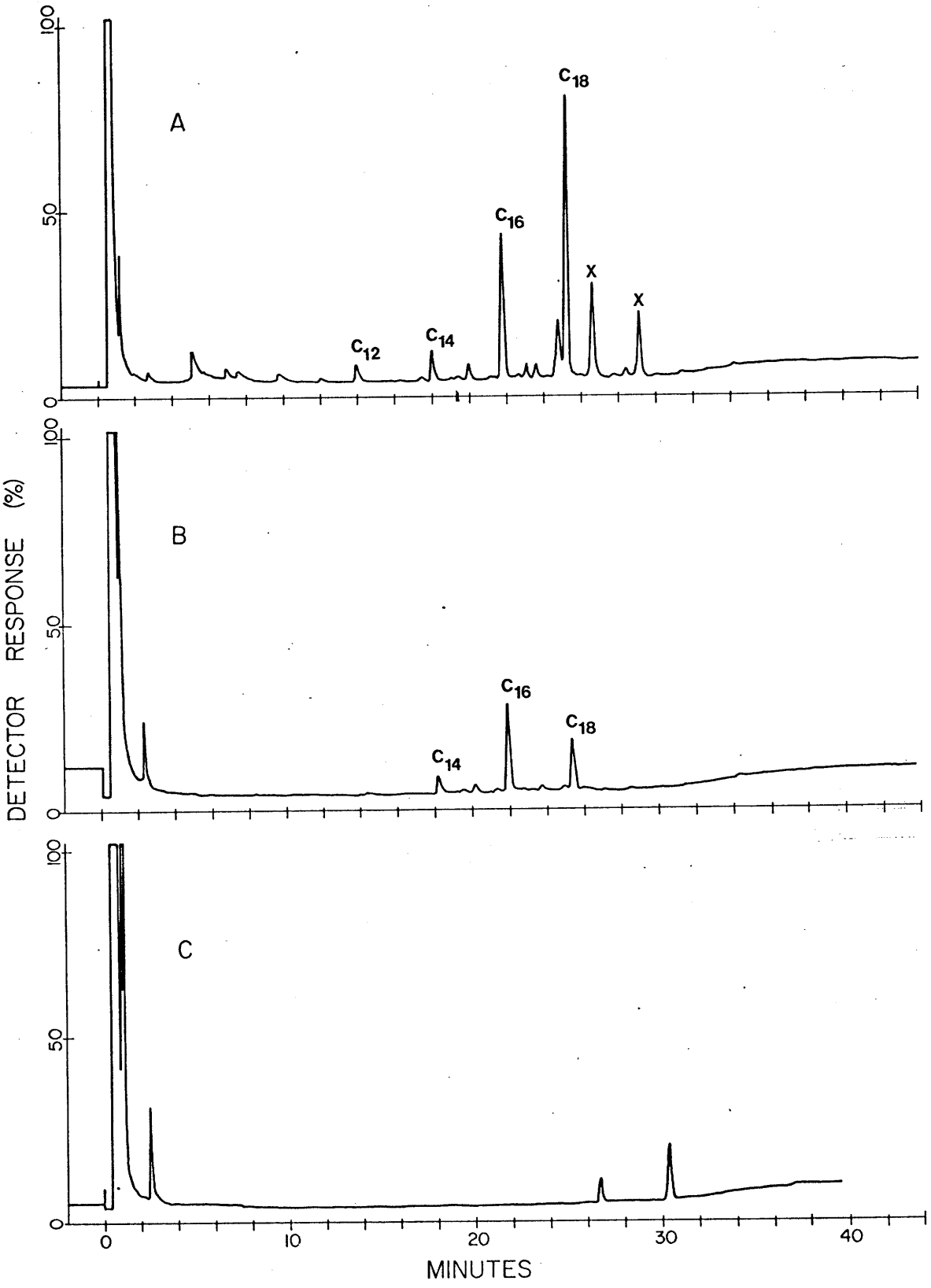
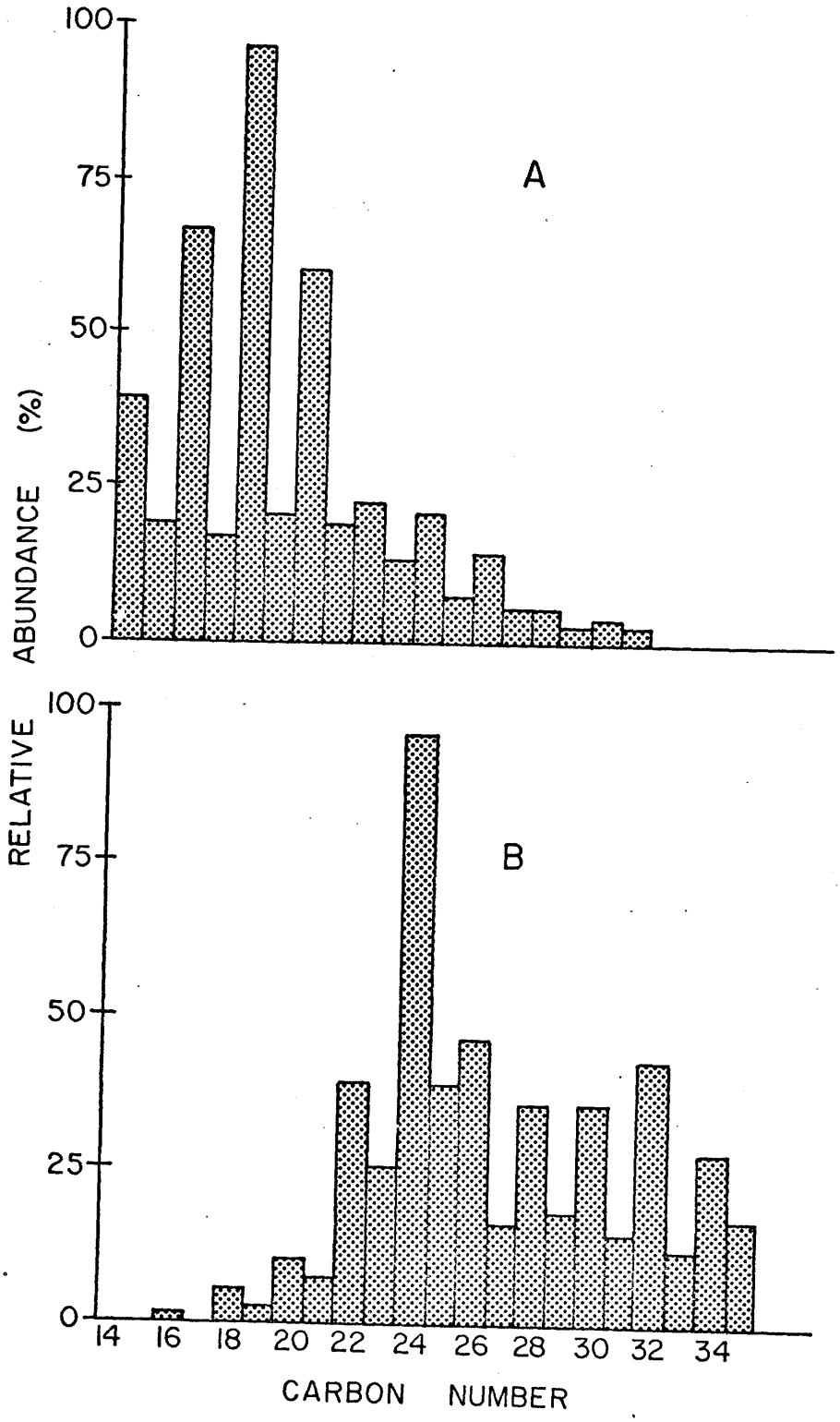

A
B
C
C_{12}
C_{14}
C_{16}
C_{18}
X
X
100
50
0
DETECTOR RESPONSE (%)
0
10
20
30
40
MINUTES

Figure 3-20. Histograms of n-alkanes in reduction products of Sargasso Sea fulvic acid (A) and normal fatty acids isolated from soil fulvic acid (Schnitzer and Khan, 1972) (B).


100
75
50
25
0
ABUNDANCE (%)
A
RELATIVE
100
75
50
25
0
B
14
16
18
20
22
24
26
28
30
32
34
CARBON NUMBER

CHAPTER 4

Introduction

This chapter presents a hypothetical structure of a typical dissolved marine humic substance. The structure is consistent with the results of this investigation and is discussed in terms of its origin, chemical and physical properties, interaction in the sea and its eventual fate.

Summary of Present Knowledge

The chemical and physical properties of seawater humic substances determined in this work (summary Table 4-1) have been discussed in Chapters 1, 2 and 3 with regard to other characterization studies of seawater organic matter (Kalle, 1966; Jerlov, 1968; Sieburth and Jensen, 1968; Kerr and Quinn, 1975; Khaylov, 1968) and of sedimentary humic substances (Nissenbaum and Kaplan, 1972; Ishiwatari, 1971; Bordovsky, 1965; Rashid and King, 1970; Rashid and King, 1967; Huc and Durand, 1974; Nissenbaum, 1974). Differences between marine and terrestrial humic substances have also been discussed (this work; Nissenbaum and Kaplan, 1972; Ishiwatari, 1974; Ishiwatari, 1973; Rashid and King, 1970; Jackson, 1975; Huc and Durand, 1974; Kerr and Quinn, 1975; Kalle, 1966; Nissenbaum, 1974) and are summarized in Table 4-2.

However, there are several similarities in the general properties of humic substances from different environments and, since the composition and environmental effects of humic substances from soils and sediments have been studied much more extensively, consideration of these similarities may be helpful in predicting unknown properties and effects of seawater humic substances. Terrestrial, sedimentary and seawater humic substances are all complex mixtures

TABLE 4-1

Summary of Sargasso Sea Fulvic Acid Characteristics

Elemental Composition:	a) C: 49.98; H: 6.40; O: 36.40; S: 0.46 b) H/C = 1.61 c) Stoichiometric formula $(C_9H_{15}O_5N)_x$
UV-VIS Spectrum:	a) Smoothly increasing absorption with decreasing wavelength b) Low extinction c) E420/665: 4.8
Fluorescence Spectrum:	a) Excitation maximum: 332 nm b) Fluorescence maximum: 405 nm (broad)
IR Spectrum:	a) Broad absorption bands b) Similar to fulvic acids from other environments c) Absorption at 1560 cm^{-1}; not in humic acid or soil humic substances d) Hydroxyl groups (Table 2-3) e) Carboxylic acid groups (Table 2-3)
$\delta^{13}C$:	-22.79 °/oo
Acid Titration:	a) Smooth curve indicates wide range of pK_a's b) Equivalent weight: 473 g
Molecular Weight Distribution:	a) 73% less than 700 b) 84% less than 1500 c) 100% less than 5000
^1H NMR Spectrum:	a) High aliphatic character b) Low aromatic character
^{13}C NMR Spectrum:	a) High aliphatic character b) Low aromatic character c) Polyhydroxyl groups d) Abundant amide, ester or acid groups e) Low ketone and aldehyde content f) Complex composition
Chemical Data:	a) Extremely complex mixture b) Polar and non-polar moieties present c) Nitrogen mainly not as hydrolyzable amino acids d) Fatty acids and other lipids are important components e) Some aromatic components

TABLE 4-2

Summary of Differences Between Lacustrine or Marine and Terrestrial Humic Substances

Character	Marine	Terrestrial	Reference
Aromaticity	low (seawater, lake sediment, marine sediment)	high	This Work Ishiwatari, 1969 Ishiwatari, 1971 Rashid and King, 1970
Phenol content	low (marine sediment)	high	Huc, 1973 Rashid and King, 1970
Nitrogen	high (seawater, marine sediment)	low	This Work Nissenbaum and Kaplan, 1972
$\delta^{13}C$ (‰)	-22 to -24 (seawater, marine sediment)	-24 to -29	This Work Nissenbaum and Kaplan, 1972
1540 to 1560 cm^{-1} ir band	present (seawater, lake sediment)	absent	Ishiwatari, 1967 This Work
Molecular weight	low (seawater) high (sediment)	high high	This Work Rashid and King, 1969
Aliphatic carbon	high (seawater)	low	This Work
UV-VIS light extinction	low (seawater)	high	This Work Kerr and Quinn, 1975

of highly functional molecules of a wide molecular weight range. They contain complex distributions of functional groups, have, by definition, the same solubility characteristics and possess some surface active character. From these considerations it is expected that both marine and terrestrial humic substances will have similar general chemical and physical effects in the environment. Indeed, both marine sedimentary and terrestrial humic substances are chelators of metal ions (Schnitzer and Khan, 1972; Koshy and Ganguly, 1969; Rashid, 1971; Rashid, 1974), growth promoting agents of plants (Schnitzer and Poapst, 1970; Prakash and Rashid, 1968; Prakash, et al., 1973), and solubilizing agents for hydrophobic compounds (Boehm and Quinn, 1973; Khan and Schnitzer, 1972; Khan, 1974).

In addition, soil humic substances are highly resistant to bacterial decomposition (Ladd, 1964; Lynch and Lynch, 1958) and they form stable complexes with clays and can, thereby, affect sorption and aggregation (Schnitzer and Khan, 1972).

Seawater humic substances have not been investigated with regard to these properties.

Hypothetical Structure

A hypothetical structure of a typical marine humic substance is presented in Figure 4-1. Its building blocks are many of the important biosynthetic molecules in the sea, such as amino acids, sugars, amino sugars, and fatty acids; they are condensed with some rearrangement (Figure 4-2). Other molecules such as carotenoid and chlorin pigments, hydrocarbons, and phenols may also be incorporated into the structure but they are not indicated here.

It is important to emphasize that the proposed structure only indicates the types of structures which are present and that the results of this work can be fully explained only by the assumption that very numerous permutations of this structure occur, ranging widely in molecular weight and in structural features. Furthermore, it is likely that various structures interact to form three-dimensional arrays which are held together through hydrogen and hydrophobic bonding, van der Waals forces, Coulombic interactions and perhaps metal chelation.

The following section demonstrates that these structural features are consistent with the present chemical and physical knowledge of marine humic substances.

Correlations with the Data

Elemental Composition

A stoichiometric formula of $(C_9H_{15}O_5N)_x$ is calculated from the seawater fulvic acid elemental composition data (neglecting sulfur). The proposed structure has a formula of $(C_{12.4}H_{18.8}O_{5.5}N)_x$ and thus the abundance of functional groups indicated is reasonable.

Molecular Weight

The molecular weight range of the three component structures in Figure 4-1 is 420 to 992. Seawater fulvic acid ranges from a few hundred to approximately 5000 in molecular weight; this requires that a continuum of larger and smaller molecules be present. Larger molecules may be more variable, cross-linked condensates; these may be important agents in the formation of

three-dimensional arrays which may incorporate smaller polymers within their tertiary structure.

Titration

Complex mixtures of many permutations of the proposed amphoteric and polyfunctional structure would display a featureless titration curve similar to that observed in this study.

UV-VIS Spectra

The proposed structure would adsorb mainly in the uv region of the spectrum: Absorption from 240 to 290 nm from the aromatic structures, absorption near 220 nm from $\pi \longrightarrow \pi^*$ transitions of carbonyl groups with some conjugated carbonyls extending to higher wavelength, weak absorption near 320 nm from $n \longrightarrow \pi^*$ transitions of carbonyl groups and some absorption extending into the visible region from $n \longrightarrow \pi^*$ transitions of conjugated carbonyls. Sufficiently complex mixtures are required to explain the smoothly increasing absorption with decreasing wavelength observed. The yellow color of these materials results from the presence of some chromophores absorbing in the blue region of the visible spectrum.

IR Spectrum

The O-H and N-H absorbance in the ir spectrum of the proposed structure would extend from 2800 to 3800 cm^{-1} because of the presence of several different functional groups (acids: 2500 to 3000 cm^{-1}; alcohols: 3450 to 3650 cm^{-1}; amines: 3300 to 3500 cm^{-1}; and amides 3100 to 3500 cm^{-1})

and because the same functions are present in different structural environments (hydrogen-bonded, conjugated, etc.), this will further broaden each absorption band. Absorbance from C-H stretching (2800-3100 cm^{-1}) results from the hydrogens on aromatic, aliphatic and other types of carbons and would also be broad. The presence of carbonyl functions in esters, acids, amides, and carboxylate anions would result in broad bands near 1700 cm^{-1} and 1560 cm^{-1} (amides and carboxylate anions). Complex absorptions in the region below 1680 cm^{-1} would result from the mixture of various C-C, C-O, C-N stretching and C-H and N-H bending vibrations. All these features are observed in the ir spectra of seawater fulvic acid.

Fluorescence Spectra

The blue fluorescence of condensation products of amino acids and sugars in model systems has been reported (Hodge, 1953), however, the source of this fluorescence in these structures is unknown. The fact that excitation maxima near 320 nm are observed in this work indicates that carbonyl $n \longrightarrow \pi^*$ absorptions may be involved.

NMR Spectra

The ^1H nmr spectrum of the proposed structure would show intense aliphatic resonances since aliphatic protons constitute about 40% of the total protons; small resonances from aromatic protons may be observed. In complex mixtures, other protons would be so variable or easily exchangeable (hydroxyl, amino, acidic protons) that they would not be easily observed.

The ^{13}C nmr spectrum would show resonances from aliphatic, aromatic, carbonyl and polyhydroxyl carbons. Again, mixtures of several variations on the proposed structure are required to explain the broad resonances.

Chemical Studies

The fact that TLC-GC fractionation is insufficient to separate the fulvic acid indicates that it is an extremely complex mixture. Furthermore, the fact that some functional groups are resistant to even drastic reduction suggests that the structures are present in hindered forms, perhaps enclosed in large, cross-linked, three-dimensional arrays. This has been proposed to explain the results of the chemical degradation of kerogen by Burlingame et al., (1969). Functional groups which are on the outer surface of these structures are easily cleaved by mild conditions but deeply buried structures require more drastic conditions which are capable of breaking-up the three-dimensional structure.

In the proposed structure (Figure 4-1) acid hydrolysis would free some amino acids while others, not linked through amide or ester bonds, would be resistant to hydrolytic cleavage. Also, hydrolysis would cleave ester and amide bonds thus forming both polar and non-polar structures. However, ether linkages and hindered ester and amide bonds (present in more complex, three-dimensional structures) would resist hydrolysis and keep most of the products in a form which would not be easily identified.

Drastic reduction and bond cleavage (e.g. by hydrogenation-bromination-hydrogenation) would cleave many bonds, and allow identification of component carbon structures. Analysis of hydrocarbon products from the proposed structure in the C_{18} to C_{32} range would reveal mainly those resulting from reduction of fatty acid components. In spite of the strong conditions used, yields of hydrocarbons were low and esters were still present in the products; this further suggests that hindered sites are present in three-dimensional structures.

Structural Features of Soil Humic Substances

Three hypothetical models of soil humic substance taken from the literature are presented in Figure 4-3. These structures are consistent with the results of degradation studies of soil humic substances. However, the lack of aliphatic components, the low H/C ratios, and the low nitrogen content in these structures are incompatable with the data obtained on seawater humic substances.

Mechanism of Formation

In the sea the most abundant sources of organic matter from which humic substances may be formed are the components of algae and zooplankton: amino acids, carbohydrates, lipids and pigments. A major precursor to soil humic substances is lignin (Hurst and Burges, 1967); but this highly aromatic material is not abundant in seawater and is, therefore, not a significant source material for seawater humic substances.

Reactions between sugars and amino acids, known as browning reactions or Maillard condensations, have been extensively studied in concentrated solutions (Hodge, 1958; Cole, 1967; Reynolds, 1969). The first step in these reactions involves the formation of a Schiff base between the amino nitrogen in amino acids and the aldehyde (or ketone) function of sugars. Subsequent reactions include rearrangements, cyclizations, and decarboxylations to form complex, brown colored mixtures referred to as melanoidins.

The formation of marine humus or Gelbstoff by a mechanism involving the condensation of amino acids and sugars has been proposed by Kalle (1966) and Nissenbaum (1974). However, the condensation of amino acids and sugars alone cannot account for the abundance of long-chain aliphatic structures in the seawater humic substances. Components such as marine lipids must also be incorporated into the products; bonding through ester or amide linkages, or reactions at sites of unsaturation forming ether linkages may be involved. In seawater organic matter is present at part per million concentrations which is not conducive to condensation reactions since they depend on the rate of intermolecular collisions. However, many processes in the sea result in high local concentrations of organic matter where such condensation reactions are feasible. For example, decaying organisms contain the precursors in high concentrations; organic films on the sea surface or on bubbles have organic matter concentrations 10 to 1000 times that of seawater (Garrett, 1972; Barger et al., 1974; Duce et al., 1972); organic matter in particles may be in

dynamic equilibrium with that in solution (Sharp, 1973); filter-feeding organisms concentrate organic matter in their guts and in fecal pellets; and surface active molecules may exist as aggregates from a few molecules to the much larger micelles and colloids (Breger, 1970; Sharp, 1973). In all these instances, organic molecules are brought within bonding distance and intermolecular reactions become possible.

Humic substances formed by these reactions may in turn serve as aggregation centers and sites of condensation. Lipids may be included in hydrophobic sites and then be bonded through condensation reactions at double bonds or through amide or ester formation at carboxyl groups. Once condensation reactions have occurred, further cross-linking and intramolecular reactions are likely to occur since functional groups are held in close proximity to each other.

Effects in Seawater

From the general structural features of seawater humic substances, predictions can be made of their properties and interactions in seawater.

Little is known about organic-metal interactions in seawater. Calculations using stability constants of simple organic compounds suggest that chelation of trace metals by organics in seawater is insignificant (Stumm and Brown, 1975). However, indirect evidence indicates that metal chelation by organics may have important effects on primary productivity in the sea by influencing the

biological availability or toxicity of trace metals (Barber and Ryther, 1968; Sunda, 1975; Martin et al., 1969). Polyacidic, polyfunctional structures are expected to be good chelators for metals since polydentate complexes are possible; this suggests that seawater humic substances have much higher stability constants than simple organic compounds and, therefore, may have significant chelation effects in seawater.

The irreversible incorporation of some trace metals by soil humic substances has been observed (Schnitzer and Khan, 1972); this may also occur with seawater humic substance and could, thereby, influence the geochemical cycling of some trace elements.

The surface activity of seawater humic substances (Table 4-1) suggests their involvement in those sea surface phenomena generally ascribed to fatty acids and other lipids (Garret, 1963, Garret, 1969; Blanchard, 1965). These include natural slicks on the sea surface, the foaming of seawater, the damping of capillary waves, and the transport of organic matter from the sea surface to the atmosphere on water droplets. Quantitatively, humic substances may be more important surface active agents since they are far more abundant than lipids in seawater. Aggregation of humic substances at the sea surface brings them in contact with the atmosphere and into direct sunlight; this may influence oxidation and oxidative cross-linking reactions of the humic substances.

The surface activity of humic substances suggests that they are adsorbed on particle surfaces in seawater. This may influence the surface charge (Niehoff and Loeb, 1974), the ion exchange reactions (Rashid, 1969) and the dissolution/precipitation reactions (Chave and Seuss, 1970) of particles in seawater. Interaction of soil humic substances with soil particles has important influences on the aggregation processes and the sorption capacity of soils (Schnitzer and Khan, 1972, p. 253).

Surface active materials can form micelles in water and can incorporate hydrophobic molecules into hydrophobic sites. Thus humic substances can increase the apparent solubility of non-polar substances in water (Boehm and Quinn, 1973; Khan, 1974; Khan and Schnitzer, 1972). Humic substances may also decrease the toxicity of organic compounds by making them unavailable to organisms (Schnitzer and Khan, 1972; Boehm and Quinn, 1973). Reactive compounds may become permanently incorporated into the humic structure through chemical bonding. Therefore, humic substances in seawater may serve as sinks of both reactive and non-polar compounds.

Fate

Organic materials have finite life times in the oceans: residence times of 1000 to 3500 years are calculated (Menzel, 1974; Williams et al., 1969; Skopintsev, 1972). These are average values and since the chemical and biological degradation and re-utilization of a large fraction of the organic matter in the sea is believed to be slow (Skopintsev, 1972; Ogura, 1972; Barber, 1968), some organic matter (e.g. humic substance) may be much older. Three mechanisms of

removal of humic substances must be considered: 1) loss to the atmosphere, 2) in situ chemical and biological degradation and re-utilization, and 3) loss to the sediment. Although humic substances may be concentrated at the sea surface and, thereby, ejected to the atmosphere on bubble droplets, transfer to land is

In the deep sea the organic matter is at low concentrations and the effects of temperature and pressure reduce bacterial activity (Jannasch et al., 1971). Therefore, the organic matter in the deep sea may be extremely stable. Indeed, investigations of the bacterial degradation of the total organic matter in deep water suggests that after 50 days, even under favorable conditions of increased concentrations and temperature, it is not re-mineralized (Barber, 1968). Most of the organic matter in the surface waters is rapidly re-utilized (Menzel, 1974; Barber, 1968; Ogura, 1972) but a small fraction is either released from organisms in or converted to more stable forms. The formation of humic substances may be, in part, responsible for this stabilization of organic carbon. Their stability is suggested by the fact that the humic substances in soil are extremely resistant to biological degradation (Lynch and Lynch, 1958; Ladd, 1964). One may speculate why humic substances are stable. Perhaps they contain toxic moieties, or the rearrangement of the organic matter has made it non-degradable by the available enzymes, or the utilizable organic matter is protected deep within humic structures whose outer surface have been degraded and become resistant.

Transport to the sediments appears to be the most important pathway for the removal of humic substances from seawater. The surface active character of seawater humic substances suggests that they are capable of adsorbing on sinking particles and thus can be transported to the sediments. In addition, dissolved organic compounds may grow in size until they become insoluble (Wangersky, 1972) and sink. Incorporation of smaller particles into fecal pellets by filter-feeding organism may provide another mechanism for transport of adsorbed humic substances to the sediments.

Humic substances are the most abundant class of organic material in modern marine sediments (Bordovsky, 1965; Degens et al., 1964; Nissenbaum and Kaplan, 1972; Wakesman, 1933). In shallow coastal waters, in situ production appears to be the predominant source of humic substances (Nissenbaum and Kaplan, 1972; Nissenbaum, 1974). However, in the deep sea, much of the organic matter may already have been present on particle surfaces in the water column before they became part of the sediment. Therefore, dissolved humic substances may be an important source of the organic matter in deep sea sediments.

Over geological time periods, humic substances are probably transformed into kerogen (Brown et al., 1972; Degens et al., 1964; Bordovsky, 1965) which is the most abundant organic material on Earth (Forsaman, 1963; Robinson, 1969; Welte, 1974). Because of the low levels of organic carbon in deep sea sediments, the organic matter probably remains dispersed throughout the sedimentary

matrix and never accumulates in concentrated organic deposits. The kerogen produced from marine humics is expected to be highly aliphatic in character. The kerogens of the Green River formation (Murphy et al., 1971; Robinson, 1969; Djuricic et al., 1972) and the Yugoslavian Aleksinac shale (Djuricic et al., 1972) are highly aliphatic in character and are the result of transformations of predominantly algal (fresh water) organic matter with little input of land derived material. In contrast, the highly aromatic humic substances of terrestrial origin (e.g. lignin) (Figure 4-3) are transformed into peat and coal (Hunt, 1964) and, where they are less concentrated, into highly aromatic kerogens such as those in the Kimmeridge shale and Australian torbanite (Djuricic et al., 1972). The ultimate fate of humic substances in sediments will depend on the depth of burial (temperature and pressure) and on the geological future of the sediment.

Figure 4-1. Hypothetical structure of seawater humic substances.

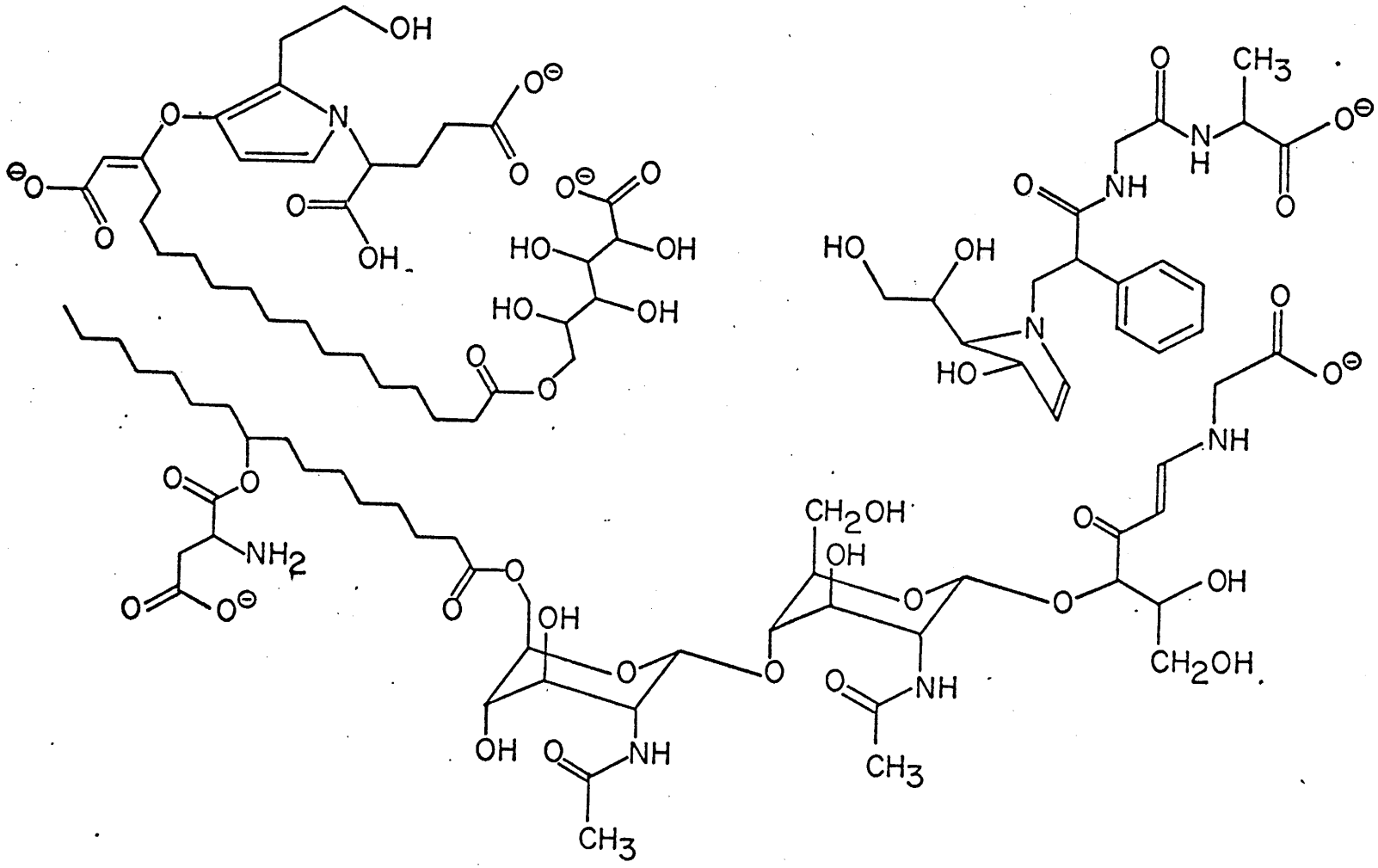


Figure 4-2. Hypothetical structure of seawater humic substances with amino acid (AA), sugar (S), amino sugar (AS) and fatty acid (FA) moieties indicated.

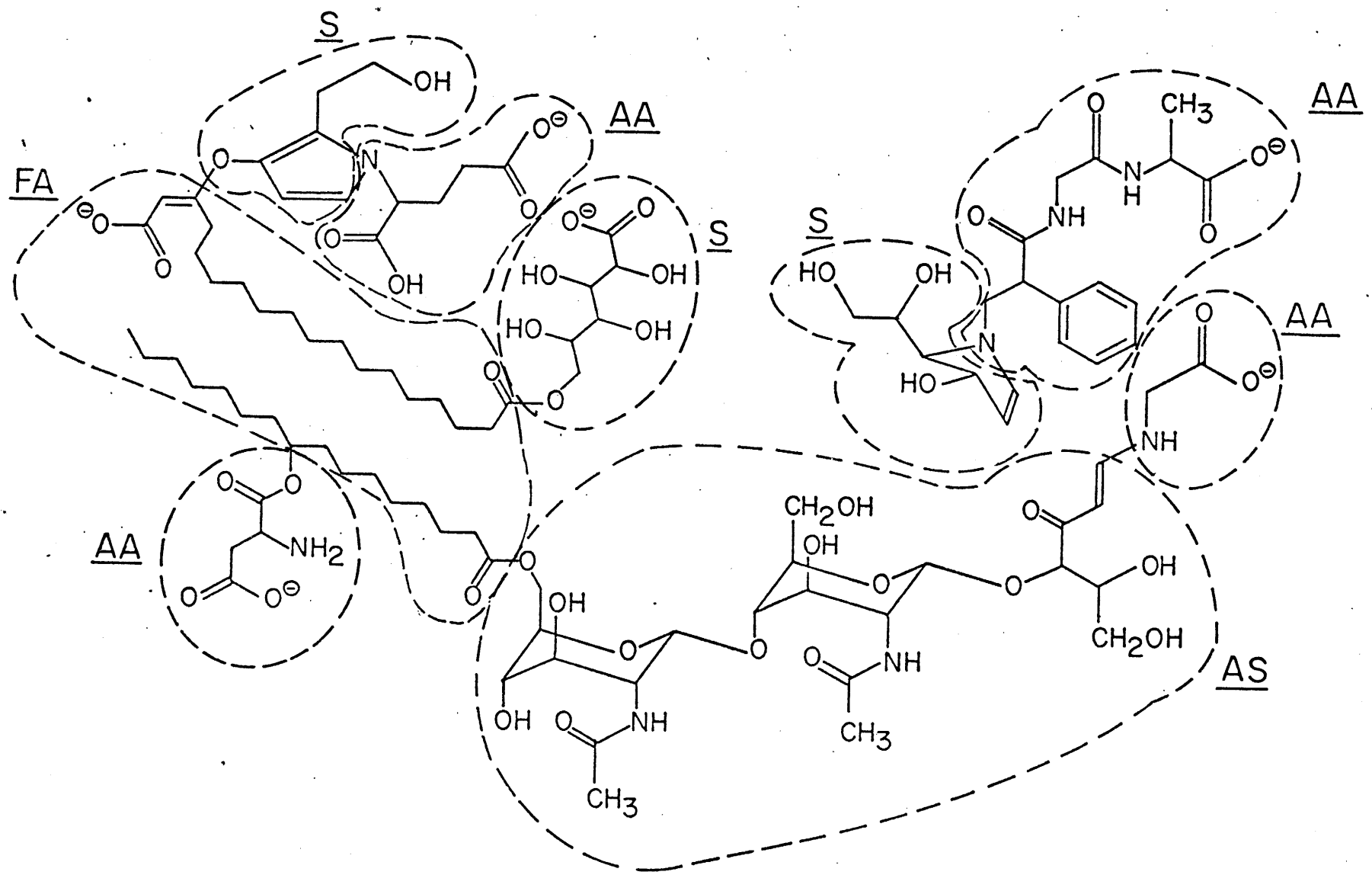
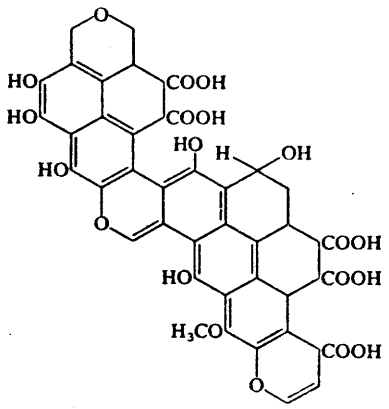
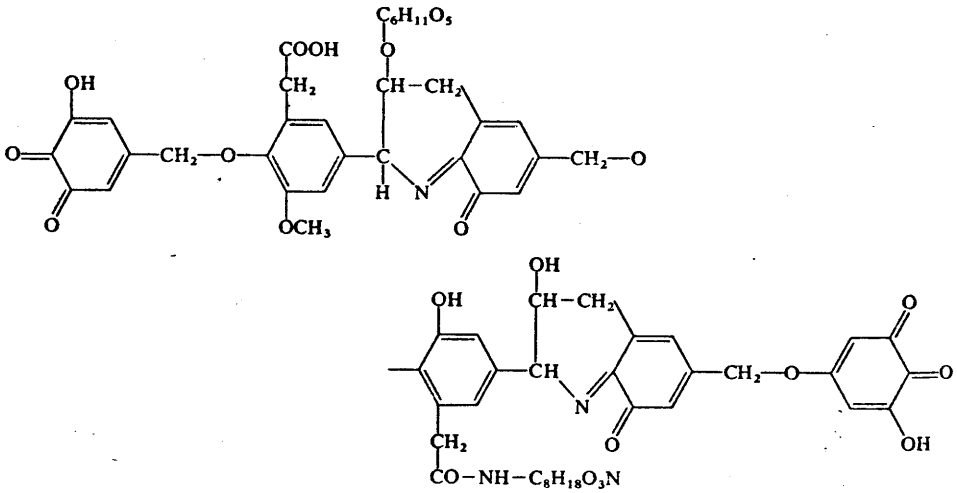

S
AA
FA
S
AA
S
AA
AA
AS

Figure 4-3. Hypothetical structures of soil humic substances proposed by Fuchs (from Stevenson and Butler, 1969) (A), Dragunov (from Stevenson and Butler, 1969) (B), and Schnitzer and Khan (1972) (C).

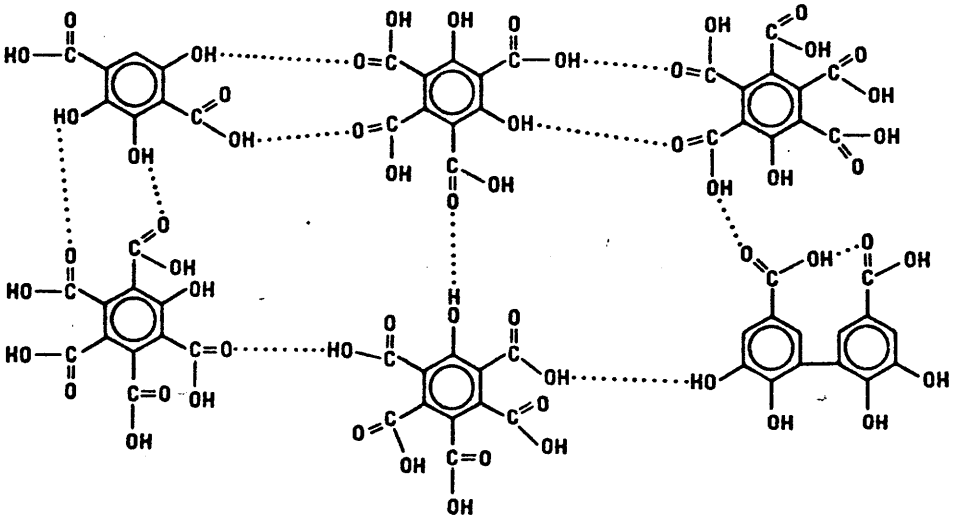
A



B



C



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